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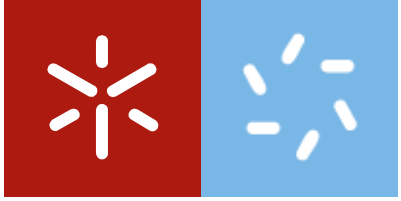

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Dissertação de Mestrado
Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho efetuado sob a orientação do
Professor Doutor Hernâni Varanda Gerós
e do
Doutor Artur Jorge da Silva Conde

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A vida é feita de batalhas, batalhas pra sobreviver

E quando falhas, vês que as falhas é que ensinam a crescer

O saber não ocupa espaço a quem abre espaço ao saber

E mesmo que chegues atrasado nunca é tarde pra aprender

Only those who will risk going too far

can possibly find out how far one can go.

Thomas Stearns Eliot

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Abstract

After detachment from the plant and until cell death, fruits remain metabolically active, resulting in compositional modifications. For certain food products, postharvest controlled stresses are applied to induce desired physical or chemical changes. In the case of grapes, a gradual dehydration process is normally utilized for raisin production and also for the production of special wines. Postharvest dehydration, in general, causes changes in texture, color, taste and nutritional value of food due to the high temperatures and long drying times required in the process. However, very limited information is so far available on the molecular modifications that are triggered during postharvest dehydration-induced compositional and phonological changes as well as on and their regulatory mechanisms.

Thus, to better understand the molecular responses and metabolic changes during grape berry postharvest dehydration, we applied an industry-mimicking slow dehydration process for eleven days at 50°C to intact berry clusters. A set of molecular, cellular and biochemical analyses were performed to assess if postharvest dehydration had a significant impact in grape berry cellular metabolism. Dried grape berries lost almost 40% of weight in water and transcriptional analyses by real time qPCR showed that several aquaporin genes (*VvPIP2;1, VvTIP1;2 and VvSIP1*) were strongly up regulated during the applied treatment. Transcriptional analysis also revealed that postharvest dehydration provoked an improvement in sugar transport capacity from berry apoplast into cells as suggested by the severe increase in transcripts of sugar transporters *(VvHT1, VvSWEET11, VvSWEET15, VvTMT1)*. As the concentration of glucose, fructose and sucrose was barely changed, post-phloem transport stimulation in berry cells could account for that occurrence. Moreover, transcriptional and biochemical enzyme activity analyses also suggested that dehydrated grape berries subjected to postharvest dehydration had substantially enhanced polyol biosynthesis capacity, as corroborated by significant sorbitol concentrations detected only in dehydrated grapes. Postharvest dehydration also affected the metabolism of organic acids in grape berries, by inducing transcriptional and biochemical activity modifications in malate dehydrogenases and malic enzymes that lead to organic acid (malate and tartrate) degradation as demonstrated by HPLC analysis. Real time qPCR analyses in key targets of the secondary metabolism *(VvSTS1, VvCHS1, VvPAL1 and VvFLS1)* suggested a general up-regulation of secondary pathways. However, a higher magnitude overexpression of the peroxiredoxin *VvPRX31*, associated with phenolics/anthocyanins degradation, could have simultaneously been responsible for the observed decrease in the concentration of total phenolics concentration in dehydrated berries. Taken together, all these molecular changes show that postharvest dehydration clearly impacts a wide range of metabolic pathways and molecular mechanisms of primary and secondary metabolism of grape berry cells.

Resumo

Após a colheita e até ocorrer morte celular, os frutos permanecem metabolicamente ativos, resultando em modificações na sua composição. Para certos produtos alimentares após a sua colheita, é utilizado um processo controlado de desidratação para induzir alterações físicas e químicas desejadas. No caso das uvas, um processo gradual de desidratação é normalmente utilizado para produção de uvas passas e para a produção de vinhos especiais e fortificados. Processos de desidratação do fruto, no global, causam alterações na textura, cor, sabor e valor nutricional dos alimentos devido a altas temperaturas e longa duração do processo. No entanto, existe escassa informação sobre as alterações metabólicas e mecanismos moleculares envolvidos na resposta a este processo.

Assim, com a finalidade de perceber melhor o metabolismo de uvas já colhidas, aplicamos um processo lento de desidratação similar ao utilizado industrialmente e colocamos cachos de uvas expostos a 50°C durante onze dias. Varias técnicas de análise molecular e bioquímica foram utilizadas para averiguar os efeitos que tratamentos de desidratação após a colheita da uva, afetam provocam no seu metabolismo. As uvas passas perderam cerca de 40% do seu peso em água e análises transcricionais por *real time qPCR* mostraram que a expressão de vários genes que codificam aquaporinas (*VvPIP2;1, VvTIP1;2* e *VvSIP1*) foi estimulada. Análises transcricionais realizadas por *real-time qPCR,* revelaram que a desidratação provocou um aumento da capacidade de transporte de açúcares do apoplasto do bago para as células, demonstrado pelo aumento dos transcritos de transportadores de açúcares *(VvHT1, VvSWEET11, VvSWEET15, VvTMT1)*. No entanto, as concentrações de glucose, frutose e sacarose permaneceram praticamente inalteradas. As uvas desidratadas apresentaram também uma síntese de polióis aumentada, comprovada pela significativa concentração de sorbitol quantificada em uvas desidratadas. Também foi visível que o metabolismo dos ácidos nas uvas foi afetado, existindo alterações transcricionais e bioquímicas em enzimas-chave que levaram a uma degradação e consequente menor concentração dos ácidos orgânicos principais (malato e tartarato), de acordo com quantificações por HPLC. Análises por *real time qPCR* a alguns genes chave do metabolismo secundário *(VvSTS1, VvCHS1, VvPAL1 e VvFLS1)* sugerem uma estimulação de várias vias de metabolismo secundário. No entanto uma sobreexpressão de maior magnitude do gene *VvPRX31*, que codifica uma peroxiredoxina, associada a degradação de fenólicos/antocianinas, poderá ter tido um papel na diminuição drástica da quantidade de fenólicos totais nas uvas passas. Em suma, todas estas alterações metabólicas demonstram que o processo de desidratação após colheita tem um forte impacto em várias vias metabólicas e mecanismos moleculares envolvidos no metabolismo primário e secundário das células do bago de uva.

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Abbreviations and acronyms

ATP	Adenosine triphosphate
C4H	Cinnamic acid 4-hydroxylase
CHI	Chalcone isomerase
CHS	Chalcone synthase
CoA	Coenzyme A
cwINV	Cell wall invertase
DAHPs	Deoxy-*D-arabino*-heptulosonate7-phosphate synthase
DW	Dry weight
DTT	Dithiothreitol
E4P	*D*-erythrose 4-phosphate
FLS	Flavonol synthase
FW	Fresh weight
HPLC-RI	High-performance liquid chromatography - refractive index detector
LDOX	Leucoanthocyanidin dioxygenase
MDH	Malate dehydrogenase
ME	Malic enzyme
OAA	Oxaloacetate
PAL	Phenylalanine ammonia-lyase
PEP	Phosphoenolpyruvate
PIPs	Plasma membrane intrinsic protein
qPCR	Real-time qualitative polymerase chain reaction
RFOs	Raffinose family oligosaccharides
SIP	Small basic intrinsic protein
STS	Stilbene synthase
TCA	Tricarboxylic acid
TIP	Tonoplast intrinsic protein
VvHT	Hexose transporter
VvSUC	Sucrose transporter
VvTMT	Tonoplast monosaccharide transporters

INTRODUCTION

1. Introduction

Grapevine (*Vitis vinifera* L.) is a woody perennial plant that predominates in Mediterranean climates and has great importance from an economic point of view as the most cultivated fruit plant in the world. According to the International Organization of Vine and Wine, there are more than 7.5 million hectares of vineyards, while 270 million hectoliters of wine is produced each year (Chaves et al. 2010; Berdeja et al. 2015; Ferreira et al. 2016).

Besides wine production, its fruit is used mostly for dietary purposes, whereas seeds and leaves are used in herbal medicine and food supplements (Fraternale et al. 2016). The grape berry is a non-climacteric fruit with relatively high sugar and moisture content very sensitive to microbial spoilage during storage after harvest. Thus, they must be consumed or processed into various products, like dried grapes, in a few weeks in order to reduce economic losses (Adiletta et al. 2016).

A gradual dehydration process (either by open sun, shade or mechanical drying) is normally utilized for raisin and high quality wine production, with particular and differentiated characteristics such as sweet and fortified wines. Raisins are rich in nutritional content and its production is presently a growing export business in many countries. According to the United States Department of Agriculture, the world raisin production is approximately of 1.3 million ton and wines from dehydrated grapes is trending up, particularly in Italy, composing a novel niche in the winemaking industry (Adiletta et al. 2016; Pangavhane and Sawhney, 2002; Wang et al. 2015; Noguerol-Pato et al. 2012).

After harvest and until cell death, fruits remain metabolically active, resulting in compositional modifications. For certain food products, postharvest controlled stresses are applied to induce desired physical or chemical changes. In general, applied dehydration causes changes in texture, color, taste and nutritional value of food due to the combination of high temperatures and water loss caused by the long drying times required in the process (Schreiner an Huyskens-Keil, 2006; Rizzini et al. 2009). These changes suggest that an applied post-harvest dehydration process might strongly influence important primary and secondary metabolic pathways of grape berry cells, such as sugar post-phloem transport and metabolism, organic acids metabolism and phenolics biosynthesis and/or degradation, which are all key metabolic pathways strongly associated with the quality of berries. However, concrete information on how the molecular mechanisms involved in these metabolic pathways are changed during post-harvest dehydration is still somewhat limited.

1.1. Grape berry development

Until the final phenological and biochemical characteristics of a fully ripe grape berry ready to be harvested and dehydrated, an immense set of biochemical and physiological processes occur during grape berry development in the plant. Grape berry growth is sustained by import of carbohydrates, mineral nutrients and water, while different components (amino acids, pigments and other compounds) are mostly synthesized within the berry. Berry development can be divided into two phases I and II separated by one lag stage, which is characterized by a slow or inexistent growth and loss of chlorophyll (Gerós et al., 2012).

During the first phase of development, which occurs after fruit-set and during the first weeks of development, there is a massive cell division and cell expansion in the pericarp of the grape. The berry is characteristically firm and green due to the high presence of chlorophyll (Conde et al. 2007).

At the beginning, there is an accumulation of tartaric acid, mostly in the periphery of the berry, while malic acid accumulates in the flesh cells at the end of this first stage. Thereafter, a second growth phase occurs that coincides with the beginning of ripening (*veraison*). This phase is characterized by softening and change in skin color of the grape berries, mainly due to an increase of synthesis and accumulation of anthocyanins. Moreover, fruit doubles in size, sugar is accumulated and there is a decline of organic acid content (Conde et al., 2007; Liang et al., 2011).

1.2. Aquaporins in grape berry

Water is by far the most abundant constituent in grape berries (75-85%) and is the main solvent of solutes like sugars, organic acids and phenolic compounds (Ribéreau-Gayon et al. 2006). Before *veraison*, berry water is provided by the phloem and the majority, by the xylem. In the final growth stage of the berries, the phloem provides almost the total of berry water requirements, however, it has to cross the biological membranes and the hydraulic conductivity of the membranes is controlled mainly by the presence of specialized proteins (aquaporins) that belong to the major intrinsic proteins (MIP) family. Aquaporins are essential in cell to cell water flow regulation and in the hydraulic conductivity of the plasma membrane and tonoplast (Alleva et al. 2006; Maurel et al. 1997; Sutka et al. 2005). For these reasons, they are very important molecular mechanisms in the response of plant cells to water deficit or dehydration, among other abiotic stresses. The MIP superfamily in plants had been originally divided into four subfamilies: the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs) and

the small basic intrinsic proteins (SIPs) (Forrest and Bhave, 2007). Recently, another subfamily called the X intrinsic proteins (XIPs) has been identified, with members in a number of dicotyledonous plants including tomato (Sade et al. 2009) and grapevine (Danielson and Johanson, 2008).

1.3. Sugar transport in grapevine

In grapevine, sugars (mostly sucrose and monosaccharides) are important for grape berry development and quality due to their role as primary carbon and energy source (Agasse et al. 2009). Besides these functions, these metabolites also play a role as osmotic and signaling molecules (Afoufa-Bastien et al. 2010) important in the plant development and responses to biotic or/and abiotic factors (Lemoine et al. 2013). For that, sugar transport is a fundamental process in which photosynthetic leaves (as sources) provide carbohydrate resources to the heterotrophic (sink) tissues, supporting their growth and development (Afoufa-Bastien et al. 2010).

Once inside the phloem, sucrose transport is motivated by hydrostatic pressure and difference of concentration between source and sink organs, like grapes, where sugars are then unloaded from the phloem (Lalonde et al. 2004). The amount of glucose and fructose accumulated over ripening is roughly similar, suggesting that sucrose is hydrolyzed by the action of invertase or sucrose synthase during this long transport to the vacuole of flesh cells (Conde et al. 2007; Agasse et al. 2009).

1.3.1. Sugar transporters

Sugar transport is mediated by a set of transporters which are implicated in basic metabolic pathways, transfer of these solutes within and between cells, and intermediate long-distance transport between tissues and organs. Sugar accumulation is a major factor in winemaking industry due to the organoleptic properties sugars confer and because they are precursors for the production of ethanol during wine fermentation (Conde et al. 2007).

After *veraison* and during ripening, berries accumulate practically equal amounts of glucose and fructose (the major soluble sugars in grape berry), suggesting that sucrose is hydrolyzed for the storage into the vacuole of mesocarp cells (Agasse et al. 2009). The allocation of sucrose into source and sink cells is controlled by sugar transporters mediating the transport of sucrose, as the case of *VvSUCs* (Howell et al. 1994; J S Hawker 1969) and also putatively by some transporters belonging to the SWEET transporters family, like *VvSWEET11* and *VvSWEET15*. Reducing monosaccharides are

also allocated into sink cells by the action of hexose transporters, as *VvHT1* (Zhang et al. 2006; Afoufa-Bastien et al. 2010). These transporters act as sugar/H^+ symporters and belong to the major facilitator superfamily in which members share a common structure of 12 transmembrane domains connected by hydrophilic loops (Chong et al. 2014). Monosaccharides are stored in the vacuole *via* tonoplast monosaccharide transporters (like *VvTMT1).*

In the mesocarp of grape berries, the phloem unloading occurs initially through a symplastic route that involves passive movement via plasmodesmata. However, an apoplastic unloading is highly suggested in the final ripening stages of the berry, when high sugar concentrations are found in the berry apoplast (Patrick, 1997; Wang, 2003). Zhang and coworkers (2006) demonstrated that a shift from symplastic to apoplastic unloading occurs at or just prior to the onset of ripening. Enzymatic hydrolysis of sucrose within sink organs by cell wall invertase (cwINV) and the uptake of residual sucrose by symporters appear to maintain simultaneously low sucrose levels and high hexose levels in the apoplast (Wada et al. 2008). Import of sucrose across the plasma membrane may occur via sucrose symporters (SUC). Sucrose can also be hydrolyzed and he hexoses resulting from this invertase activity (glucose and fructose) are then be transported across the plasma membrane of storage cells by monosaccharide transporters, like the hexose transporters *VvHTs*, and subsequently stored in vacuole through tonoplast monosaccharide transporters (TMTs) as VvTMT1 (Figure 1).

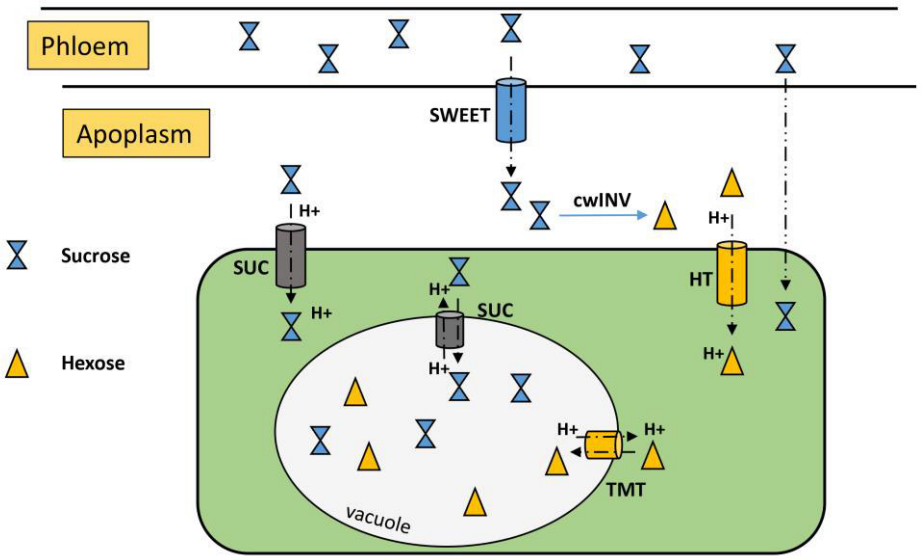

Figure 1. Speculative model for sugar uptake into berries after veraison. Single, yellow triangles represent hexose molecules, double, blue triangles represent sucrose. SUC, sucrose; SWEET,

SWEET transporter; cwINV, cell wall invertase; SUC, sucrose transporter; HT, hexose transporter; TMT, tonoplast monosaccharide transporters.

1.3.2. Polyols in grape berry

Plants have developed complex biochemical and physiological systems to mitigate the negative effect of abiotic stresses. Present in all living forms (Noiraud et al. 2001), polyols (or sugar alcohols) are an example of organic molecules accumulated that protect the cells against harmful osmotic and metabolic imbalances caused by different stresses (Pillet et al. 2012; Agasse et al. 2009). Sugar alcohols are closely related to sugars, as they represent the chemically reduced form of an aldose or ketose sugar.

Mannitol, formed by six carbons, is the most abundant polyol in nature and its accumulation is essential for salt/osmotic stress tolerance in *Olea europaea* and for the protection against heat stress-induced oxidative damage and excessive solar irradiance (Conde et al. 2011, 2015). Sorbitol is other polyol commonly found in nature, is a polyhydric alcohol with sweetness properties. In higher plants, is synthesized in mature leaves from glucose-6-phosphate by the consecutive activities of an aldose-6-P-reductase (Negm and Loescher, 1981) and a specific phosphatase. Sorbitol accumulation also contributed to increased salt/water deficit tolerance in various fleshy fruits (Conde et al. 2015). In grapevine's mannitol and sorbitol metabolism, the NAD-dependent enzymes mannitol dehydrogenase (MTD) and sorbitol dehydrogenase (SDH) are crucial in regulating the pool of these polyols, as they are responsible for catalyzing their oxidation. Also, in grapevine berries, galactinol and raffinose family oligosaccharides (RFOs) are compatible solutes and additionally may act as signaling molecules that mediate stress responses. RFOs are synthesized from sucrose by subsequent additions of activated galactose moieties donated by galactinol. (Pillet et al. 2012).

1.4. Organic acids in the grape

Organic acids are present in all plants, supporting various and different aspects of cellular metabolism (Sweetman et al. 2009). Together with sugars, they represent the main soluble constituents of ripe fruits and have great influence in the organoleptic properties (flavour, color and aroma) of wines and grape juices (Mato et al. 2005). Moreover, organic acids metabolism has effect on the stability of fruit beverages, reduce microbial spoilage and lessen oxidation due to the low-pH

conditions. Immature berries are also preserved from early predation by their unpalatability to birds and other aggressors (Ford, 2012).

The majority of organic acids present in the fruits is synthesized in the flesh from imported sugars (Sweetman et al. 2009; Etienne et al. 2014) and can be used in the Krebs cycle (respiration), gluconeogenesis, fermentation to ethanol, amino acid synthesis/interconversion, and as a substrate for the synthesis of secondary metabolites such as pigments (Famiani et al. 2015).

Environmental factors and cultivation practices affect this metabolism and the organic acid content in flesh of fruits (e.g. temperature, light intensity, cultivar, rootstock, mineral nutrition, water availability, fruit load/pruning). However, how these modifications occur is in most cases uncertain (Etienne et al. 2014).

The sourness is generally attributed to proton release from acids, while the anion forms each contribute with a distinct taste. Acidity is also one of the main ripening indicators that determines the harvest date, particularly for fruits in which a small level of acidity is important for further processing (Sweetman et al. 2009; Johanningsmeier et al. 2005).

The variety of organic acids and the levels to which they accumulate are extremely variable and depend of species, developmental stages and tissue types (Walker et al. 2011). Berries of grapevine *V. vinifera* are particularly unusual because accumulate significant concentrations of tartaric and malic acid (despite progressive reduction in malate content during ripening), by far the predominant acids at all stages of development, that contribute to the pH and acidity of the grape beverages (Sweetman et al. 2014; Ford, 2012).

1.4.1. The importance of malic acid and its metabolism

Malic acid is an important plant metabolite, it is likely present in all cell types and is the principal acid in many fruits, both climacterics (like tomato, peach, apple and banana) and non-climacteric (as pineapple, lime, cherry and grape) (Sweetman et al. 2009). It has, moreover, showed influence over the stomatal aperture, improving plant nutrition and increasing resistance to heavy metal toxicity. Malate can also affect characteristics on wine through involvement in secondary processes such as carbonic maceration and malolactic fermentation, and can even alter the growth capabilities of malolactic bacteria (Schulze et al. 2002; Fernie and Martinoia 2009; Lee et al. 2008; Kunkee, 1991).

Malic acid is (after tartrate, that is not used in primary metabolic pathways,) the major organic acid accumulated during early berry development and the only high-proportion organic acid actively metabolized throughout ripening of grape berry (Sweetman et al. 2009). Before *veraison*, grapes accumulate this acid mostly through the metabolism of sugars translocated to the berry. In a post-*veraison* fruit, malate is catabolyzed by many pathways, like TCA cycle and respiration, gluconeogenesis, amino acid interconversions, ethanol fermentation, and the production of complex secondary compounds such as anthocyanins and flavonols (Famiani et al. 2000; Farineau, 1977; Ruffner, 1982). With the accumulation of sugars and inhibition of glycolysis in ripening grapes, malate is likely a vital source of carbon for these pathways (Ruffner and Hawker, 1977).

The enzymes NAD- or NADP-MDH are present in several cellular compartments. MDHs predominantly catalyze the reversible reduction of oxaloacetate to malate and are important in several metabolic pathways (Nunes-Nesi et al. 2007; Sweetman et al. 2014). However, if malate is abundant and OAA is further metabolized into compounds as PEP or aspartate, then MDH activity will favor the conversion of malate to OAA. Still, while the MDH reaction is reversible, affinities of the mitochondrial (mMDH) and cytosolic (cytMDH) enzymes are higher for NADH and OAA than for NAD^+ and malate, therefore favoring the synthesis of malate *in vitro* (Sweetman et al. 2009; Taureilles-Saurel et al. 1995). Malic enzyme (ME) catalyzes the reversible reaction between malate and pyruvate, it is activated by fumarate and coenzyme A (CoA) and is potentially regulated via changes in aggregation state. The enzyme is formed by two subunits with similar primary sequence and *in vitro* studies have provided evidence that both subunits are required for activity. Malic enzyme can exist in a different of oligomeric forms (heterodimer, heterotetramer, and heterooctamer) and each has distinct kinetic properties (Day et al. 1984; Grover S. D and Wedding R. T, 1982). Depending on the isoform present, cellular conditions and the availability of substrates, is potentially involved in both malate synthesis and degradation. While NADP-ME is reversible, it directionality favors malate degradation, and is dependent on post-translational regulators, including $NAD(P)^+$/NAD(P)H ratio, substrate availability and pH conditions (Franke and Adams, 1995). Furthermore, an intensification of NADP-ME activity has been observed during the ripening of grape berries (J.S. Hawker, 1969; Ruffner et al. 1976; Ruffner et al. 1984), supporting a role for this enzyme in malate degradation.

1.5. Phenolic compounds in grape berries

Secondary metabolites have a fundamental role on plant defense mechanisms against biotic or abiotic stresses and are also important for plant growth and development. In grapevine, secondary metabolism has tremendous influence in wine production, as they contribute to color, flavor, aroma, texture, astringency, stabilization of wine and exhibit antioxidant properties (Costantini et al. 2006).

Grape berries accumulate a vast collection of phenolic compounds, mostly polyphenols, that differ according to the varieties and the environments where the cultivar grows. The composition of grape berries strongly affects the properties and sensory qualities of wine, particularly phenolics, that also have beneficial effects in many aspects of human health (Corder et al. 2006; Jang et al. 1997). Phenolic compounds consist in molecules with one or more hydroxyl (OH) substituents bonded to a six-carbon (C6) aromatic ring. They are divided in two major groups, nonflavonoid phenolics and flavonoids (Teixeira et al. 2013).

1.5.1. Nonflavonoids

Nonflavonoids include hydroxybenzoic acids, hydroxycinnamic acids, volatile phenols and stilbenes. Except for hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic acids), that are the third most abundant class of soluble phenolics, nonflavonoids are present in berry in low concentration (Adams, 2006). Mostly founded in the free form, the major hydroxybenzoic acids in grape are gentisic, salicylic, gallic and *p*-hydroxybenzoic acids (Pozo-Bayón et al. 2010). Gallic acid is the most represented hydroxybenzoic acid, which is found free and conjugated with acyl substituent of flavan-3-ols. Other benzoic acids such as protocatechuic, vanillic, and syringic acids were reported in Riesling wine from Germany (Baderschneider and Winterhalter, 2001).

Stilbenes are low molecular weight phenolics present naturally in a large number of edible plants, including several species of genus *Vitis*, like *V. vinifera.* Their biosynthesis increase from *veraison* to ripening and possess antifungal activity, allowing plants to deal with pathogen attacks (Langcake and Pryce, 1977; Bavaresco et al. 2009). Some stilbenes present in grape berries, particularly resveratrol, have been long studied and known for their benefits to human health (Aggarwal and Shishodia, 2006).

1.5.2. Flavonoids

Flavonoids are C6-C3-C6 polyphenolic compounds, with two hydroxylated benzene rings (A and B) connected by a tree carbon chain that is part of a heterocyclic C ring (Figure 2). Depending of the oxidation state of the C ring, flavonoids can be divided in flavonols, flavan-3-ols and anthocyanins (Downey et al. 2003). The composition and concentration of flavonoids in grapes is highly variable and modulated by external biotic and abiotic factors (Bavaresco et al. 2009).

Flavonols are products of the flavonoid biosynthetic pathway, further described ahead, and are mainly synthesized in the grape skin. Their most widespread roles in plant appear to be as UV protectants localized in the upper epidermis and as copigments with the anthocyanins in flowers and fruit (Mattivi et al. 2006; Downey and Rochfort, 2008). Copigmentation is an association between flavonols and the anthocyanin pigments that confers stability on the coloured form of the anthocyanin molecule resulting in increased colour (Downey et al. 2003). Of the three main flavonols, kaempferol, quercetin and myricetin, mainly quercetin-3-O-glucoside and -3-O-glucuronide are found in grape berries (Price et al. 1995).

Flavan-3-ols are the most abundant phenolics in grape berries and various terms have been used to describe these compounds in the literature, however proanthocyanidins and condensed tannins (their polymeric form) are used more frequently (Aron and Kennedy, 2008). Flavan-3-ols are characterized by the presence of a hydroxyl group at the 3 position of the C ring (Figure 2) and are detectable in every part of the berry, despite being more concentrated in seeds, followed by skin and in a lesser extent in berries' flesh. Nowadays, the general role of these compounds in plants is related to their protection against harmful intruders such as microbes, fungi, insects and herbivorous animals, due to their astringent properties (Aron and Kennedy, 2008; Verries et al. 2008; Pfaffl, 2001; Mattivi et al. 2006). Proanthocyanidins have also influence in wine properties and are responsible for the grape skin organoleptic properties such as astringency and bitterness (Teixeira et al. 2013).

Anthocyanins in berries comprise a large group of water-soluble pigments and are found mainly in the external layers of the hypodermis (the skin) (Szajdek and Borowska, 2008). They are derivatives of the flavylium cation and are synthesized from anthocyanidins by glycosylation at the 3 and 5 positions of the C ring (Janvary et al. 2009). These secondary metabolites are responsible for the pigmentation red, blue and purple of plant tissues. Anthocyanins play an important role in plant reproduction, by attracting pollinators and seed dispersers, and also in protection from photo-

oxidative stress (Winkel-Shirley, 2002). In grapes, the large amounts of anthocyanins accumulated, contribute to the sensory attributes of wine. Furthermore, considerable attention has been paid to the health benefits of anthocyanins, since epidemiological investigations have indicated that the moderate consumption of anthocyanin-rich products such as red wine is associated with a lower risk of cardiovascular disease (Hou, 2003; Mori et al. 2007) Anthocyanins are synthesized in the cytosol of the epidermal cells and then stored in the vacuole (Fontes et al. 2011; Braidot et al. 2008).

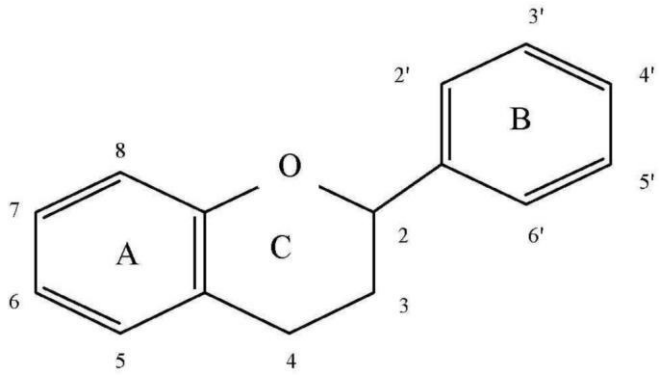

Figure 2. Flavonoid C6-C3-C6 skeleton. Two hydroxylated benzene rings (A and B) connected by a three carbon chain that is part of a heterocyclic C ring.

1.5.3. Phenolic biosynthesis

The amino acid phenylalanine is the precursor of all phenolics through the phenylpropanoid and flavonoid pathways. Phenylalanine derive from the shikimate pathway, which connects carbohydrate metabolism with the biosynthesis of aromatic amino acids and secondary metabolites (Sparvoli et al. 1994).

Phenylpropanoids contribute to all aspects of plant responses towards biotic and abiotic stimuli. They are indicators of plant stress responses upon variation of light or mineral treatment and are also key mediators of the plants resistance towards pests (La Camera et al. 2004). Phenylpropanoid-based polymers, like lignin, suberin, or condensed tannins, contribute substantially to the stability and robustness of gymnosperms and angiosperms towards mechanical or environmental damage, like drought or wounding (Vogt, 2010).

1.5.3.1. Biosynthesis of phenylpropanoids

In the phenylpropanoid pathway, three enzymatic transformations redirect the carbon flow from primary metabolism converting phenylalanine into 4-coumaroyl-Coa. Firstly, deamination by phenylalanine ammonia-lyase (PAL) forms the phenylpropanoid skeleton, producing cinnamic acid. Cinnamic acid 4-hydroxylase (C4H) catalyzes the introduction of a hydroxyl group at the *para* position of the phenyl ring of cinnamic acid, producing *p*-coumaric acid. The carboxyl group of *p*-coumaric acid is then activated by formation of a thioester bond with CoA, a process catalyzed by *p*-coumaroyl:CoA ligase (4CL), forming 4-coumaroyl-CoA.

Stilbene and flavonoid pathways begins with the end product of phenylpropanoid pathway, 4-coumaroyl-CoA, that is substrate to both stilbene synthase (STS) and chalcone synthase (CHS) (Figure 3). Various phenylpropanoid pathway intermediates are also diverted into biosynthetic pathways for benzoic acid, salicylic acid, and coumarins.

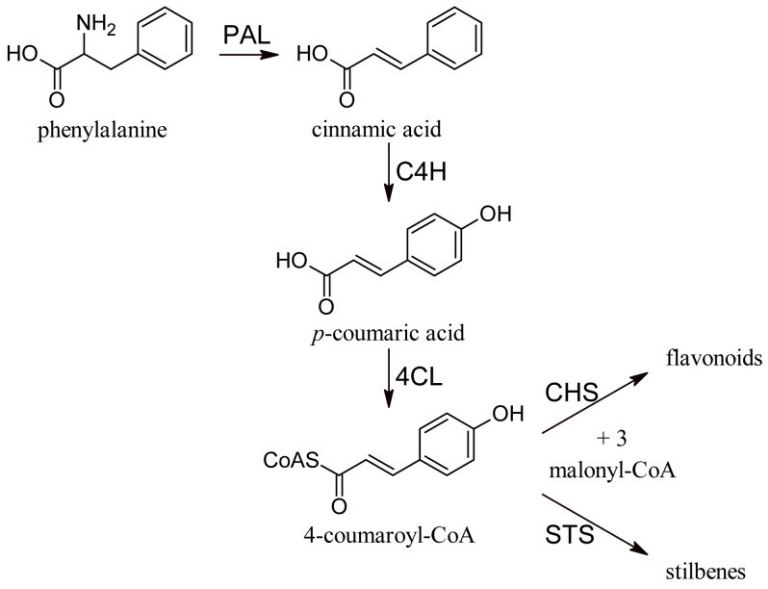

Figure 3. Phenylpropanoid pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; STS, stilbene synthase (Flamini et al. 2013).

1.5.3.2. Flavonoid pathway

The biosynthesis of flavonoids (Figure 4) is the culmination of two metabolic pathways, the shikimate's (that has phenylalanine as product) and the phenylpropanoid's.

Chalcone synthase (CHS) is responsible for the first step of the flavonoid pathway, synthetizing tetrahydroxychalcone from one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA. Chalcone isomerase (CHI) ends the formation of the molecular structure to produce naringenin, which may undergo different hydroxylations through various enzymes forming dihydroflavonols. This dihydroflavonols serves as subtracts to the flavonol synthase (FLS) that catalyzes the formation of the flavonols kaempferol, quercetin, and myricetin. The formation of anthocyanins and flavan-3-ols also derives from dihydroflavonols (Gerós et al. 2012). Firstly, dihydroflavonols are reduced into flavan-3,4-ols (leucoanthocyanidins) that are posteriorly converted into anthocyanidins by the enzyme leucoanthocyanidin dioxygenase (LDOX) (Gollop et al. 2001).

Anthocyanidins are unstable and can be stabilized by a glycosylation reaction, catalyzed by UDP-glucose flavonoid 3-*O*-glucosyltransferase (UFGT), which results in anthocyanin production and is the last biosynthetic step of anthocyanin synthesis and the final part of the flavonoid pathway.

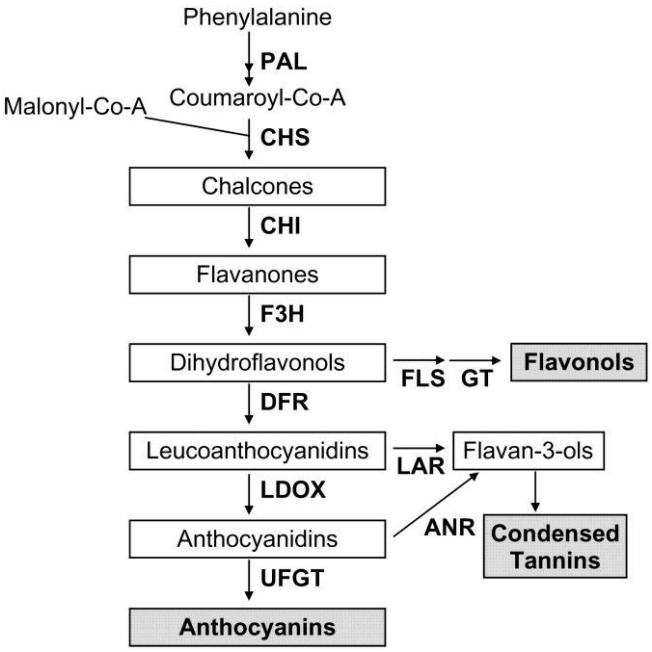

Figure 4. Simplified diagrammatic representation of the flavonoid biosynthetic pathway. Enzymes for each step are shown in bold. Flavonoid intermediates are boxed and principle flavonoid end products are in gray boxes. CHS, Chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3β-hydroxylase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase; UFGT, UDP-glycose:flavonoid-3-O-glycosyltransferase for anthocyanin synthesis. The unidentified enzyme encoding a glycosyl transferase for flavonol glycone synthesis is referred to as GT (Takos et al. 2006).

1.5.3.3. Stilbene Pathway

Plant stilbenes are derived from the general phenylpropanoid pathway. All higher plants seem to be able to synthesize malonyl-CoA and CoA-esters of cinnamic acid derivatives, but only few plant species are able to produce stilbenes (Chong et al. 2009).

Stilbene synthase (STS) is characteristic of stilbene-producing plants and controls the first step of the stilbene pathway, competing with CHS for the same substrate, 4-coumaroyl-CoA. STS catalyzes three reactions of condensation of 4-coumaroyl-CoA with 3 molecules of malonyl-CoA producing resveratrol. This resveratrol has a different ring folder, compared to tetrahydroxychalcone, the product of CHS, since the terminal carboxyl group is removed prior to closure of the A ring.

Objectives

As mentioned above, postharvest dehydration is a stressing event that induces significant modifications in the metabolism of fruits, affecting total amounts of compounds essential to the quality of berries, including sugar, phenolics, organic acids, amino acids, mineral elements, and pigments. Unlike preharvest water stress, limited information is available on the specific processes triggered by typically industrial postharvest dehydration of grape berries and the regulatory mechanisms involved in these changes.

Given that, the purpose of this study was to analyze particular metabolic changes during this process using molecular and biochemical analyses to understand the role of sugar transporters, which are involved in sugar allocation from berry apoplast into the cells, aquaporins (VvSIP1 and VvXIP1), and polyol transporters during the dehydration process. Moreover, the metabolism of organic acids and secondary metabolites (phenolics – phenylpropanoids, stilbenes), due to their relevance in berry/wine flavor; and of polyols, due to their involvement in water deficit stress tolerance, were also thoroughly investigated.

To accomplish these objectives, we recurred to a combination of several techniques including RNA extraction and transcriptional analyses by real-time qPCR, protein extraction and enzymatic activity measurements, and metabolite (sugars, acids, polyols, phenolics, anthocyanins) extraction and quantification by HPLC among other biochemical and cell biology approaches.

MATERIALS AND METHODS

2. Material and Methods

2.1. Grapevine field conditions and sampling

Clusters of *Vitis vinifera* cv. Sémillon, a golden-skinned grape used to make dry and sweet white wines, mostly in France and Australia, were harvested from a commercial vineyard in Fafe, north of Portugal. In this region the climate is typically Mediterranean, with a warm temperate climate, dry and hot summers, and with higher precipitation during autumn and winter (Kottek et al. 2006). Vineyard was managed without irrigation and grown following standard cultural practices applied in commercial farms.

The grape clusters (sound berries and uniform size) were randomly, carefully and representatively harvested and, subsequently, a set of grape clusters were placed in small perforated boxes where they were subjected in laboratorial conditions to an industry-mimicking slow dehydration process at 50°C for eleven days, while other set was immediately frozen in liquid nitrogen (control). Sampling was performed after five days and eleven days of dehydration, by collecting randomly and representatively berries from the dehydrated clusters and immediately freezing them in liquid nitrogen. None of the clusters or individual berries presented signs of fungal contamination. Berries were ground to a fine powder under liquid nitrogen refrigeration and stored in -80°C for posterior studies.

2.2. Quantification of total phenolics and anthocyanins

The concentration of total phenolics and anthocyanins was performed as described in our previous work (Conde et al. 2016). Briefly, the concentration of total phenolics was quantified by the Folin-Ciocalteu colorimetric method in berries from all experimental conditions. Total phenolics were extracted in 1.5 mL of pure methanol from 100 mg of berry grounded tissue. The homogenates were vigorously shaken for 15 min and subsequently centrifuged at 18000 *xg* for 20 min. Twenty μL of each supernatant were added to 1.58 mL of deionized water and 100 μL of Folin reagent, vigorously shaken and incubated for 5 min in the dark before adding 300 μL of 2M sodium carbonate. After 2 h of incubation in the dark, the absorbance of the samples was measured at 765 nm. Total phenolic concentrations were estimated using a gallic acid calibration curve and represented as gallic acid equivalents (GAE). Anthocyanins were extracted from 150 mg of grape berry grounded tissue with 1 mL of 100% acetone. The suspension was vigorously shaken

for 30 min. The homogenates were centrifuged for 20 min at 18000 *xg* and the supernatants were collected. Anthocyanin extracts were diluted 1:10 in 25 mM potassium chloride solution pH 1.0 and absorbance was measured at 520 nm and 700 nm, using 25mM potassium chloride solution pH 1.0 as blank. Total anthocyanin quantification was calculated in relation to cyanidin-3-glucoside equivalents, calculated by equation 1, per DW:

$$[Total\ anthocyanins](mg/L) = \frac{(A_{520}-A_{700})\times MW\times DF\times 1000}{\varepsilon\times 1} \quad (1)$$

where MW is the molecular weight of cyanidin-3-glucoside (449,2 g mol^{-1}), DF is the dilution factor and ε is the molar extinction coefficient of cyanidin-3-glucoside (26900 M^{-1} cm^{-1}).

2.3. RNA extraction

A total of 200 mg of grape berry tissue previously grounded in liquid nitrogen was used for total RNA extraction following the protocol by Reid and coworkers (2006) in combination with purification with RNeasy Plant Mini Kit (Qiagen). After treatment with DNase I (Qiagen), RNA integrity was confirmed running the samples in 1% agarose gel stained with SYBR Safe (InvitrogenTM, Life Technologies). cDNA was synthesized from 1 µg of total RNA using Omniscript Reverse Transcription Kit (Qiagen).

2.4. Transcriptional analyses by real-time qPCR

Real-time PCR analysis was performed with QuantiTect SYBR Green PCR Kit (Qiagen) using 1 µL cDNA (diluted 1:10 in ultra-pure distilled water) in a final reaction volume of 10 µL per well. For reference gene, *VvGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was selected, as this gene was proven to be very stable and ideal for qPCR normalization purposes in grapevine (Reid et al. 2006). Specific primer pairs used for each target or reference gene are listed on Table 1. Melting curve analysis was performed for specific gene amplification confirmation. The expression values were normalized by the average of the expression of the reference genes as described by Pfaffl (2001). For all experimental conditions tested, one or two independent runs with triplicates were performed.

Table 1 Primers forward (F) and reverse (R) used for gene expression analysis by real-time qPCR.

Gene	Acession Number (Genoscope)	Primers	Ref. Primers
VvCHS1	GSVIVT00037967001 (8x)	F: 5′-CGAGCTCACCACCGAGCACCTTACCT-3′	Boubakri et al. (2013)
		R: 5′-CCGCTCGAGTGTTGGCTACCTGCTTCACT-3′	
VvFLS1	GSVIVT00015343001	F: 5'-CAGGGCTTGCAGGTTTTTAG-3'	Boubakri et al. (2013)
		R: 5'-GGGTCTTCTCCTTGTTCACG-3	
VvGAPDH	GSVIVT00009717001	F: 5'-CACGGTCAGTGGAAGCATCAT-3'	Conde et al. (2014)
		R: 5'-CCTTGTCAGTGAACACACCAG-3	
VvcytMDH	GSVIVT01028332001	F: 5'-TGGAACTCCAGAGGGAACTTGG-3'	Sweetman et al. (2009)
		R: 5'-TCTTCCATTCTCCAGCACAGCAAG-3'	
VvmytMDH	GSVIVG01021185001	F: 5'-TGTACCAGTTGTTGGTGGTCACG-3'	Sweetman et al. (2009)
		R. 5'-TGTTGGATTTCGGTGTGGCTTG-3'	
VvmytME	GSVIVT01026824001	F: 5'-TTCTCGAAGATCGCCTTTGTGG-3'	Sweetman et al. (2009)
		R: 5'-TGAACTCCAAGGTCACCCAGAC-3'	
VvPIP2;1	GSVIVT01016276001	F: 5'-TCAAGGCTCTTGGGTCATTCAGG-3'	Fouquet et al. (2008)
		R: 5'-CAATTGGAAGAGGTGCCAGAACTC-3'	
VvTIP1;2	GSVIVT01033677001	F: 5'-TTCGTGATCGCTGATCTTTCCAC-3'	Fouquet et al. (2008)
		R: 5'-CAAACGCGTTCCACACTGTCAC-3'	
VviPRX31	VIT_14s0066g01850 (UniProt)	F: 5'-ATGGCATTGATCCTCTTTTC-3'	Movahed et al. (2016)
		R: 5'-CTAGTTTAAGGCATCACACC-3'	
VvPLT1	GSVIVT01031930001	F: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGAGATGGCTACAGGGAA-3'	Conde et al. (2014)
		R: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCATCCATTAGTTCCTAATTGAATC-3'	
VvTMT1	GSVIVG01009024001	F: 5'-GTTGCCGTCAACTTCGCAAC–3	Hayes et al. (2007)
		R: 5'-GAAGGAATTTAGCTATGGCAGAG-3'	
VvSWEET11	GSVIVT01010993001	F: 5'-GGGACGTGCATAGAAGCTACA-3'	Chong et al., (2014)
		R: 5'-GCAGACCCAACCGACTATCTT-3	
VvGOLS1	GSVIVT01028174001	F: 5′-TGATTACAGCAGTGTTTTGCC-3	Pillet et al. (2012)
		R: 5′-CGAGAGTACTGGCCTCTTCTAG-3′	
VvcwINV	GSVIVG01016869001	F: 5'-ATGAATCATCTAGTGTGGAGCAC-3'	Hayes et al. (2007)
		R: 5'-CTTAAACGATATCTCCACATCTGC-3	
VvPAL1	GSVIVG01025703001	F: 5′-CCGAACCGAATCAAGGACTG-3	Boubakri et al. (2013)
		R: 5′-GTTCCAGCCACTGAGACAAT-3′	

VvSTS1	GSVIVT01010590001	F: 5′-CGAAGCAACTAGGCATGTGT-3	Boubakri et al. (2013)
		R: 5′-CTCCCCAATCCAATCCTTCA-3′	
VvHT1	GSVIVG01003181001	F: 5'-TCGGAGTGGATGGAGAACCTTG-3'	Hayes et al. (2007)
		R: 5'-GACATCACCACCACAAAGAAGGC-3'	

2.5. Major Sugars and organic acids extraction and quantification by HPLC analyses

The extraction of sugars and organic acids from grapevine berry samples was adapted from a method described in (Eyéghé-Bickong et al. 2012). Extracts were obtained adding 800 µL of dH_2O and 5% (w/v) insoluble PVPP to 80mg of grape berry frozen powder and by vigorously vortexing. An equal volume of chloroform (800 µL) was added to the mixture and the biphasic solvent was vortexed for 5 min to mix and incubated at 50ºC for 30 min with continuous shaking. After incubation, the samples were centrifuged at 17,500 xg for 10 min at room temperature to recover the upper aqueous phase containing the sugars and organic acids. The aqueous phase was re-centrifuged (as above) to remove any residual cell debris. The supernatant was transferred to HPLC vials, after filtration, and crimp-sealed for HPLC analysis. Each grapevine sample was extracted in triplicate before HPLC analysis. Chromatographic analyses were carried out on a Hitachi Auto Sampler L-2200 Elite LaChrom chromatograph coupled to a Refractive Index (RI) detector. The injections were of 20 µL and the flow rate was kept constant throughout the analysis at 0.5 mL min^{-1} at 60°C. The HPLC column was a Rezex RCM monosaccharide Ca^{2+} (8%) and with water as the mobile phase. Sugar, sorbitol and tartrate concentrations on each sample were determined by comparison of the peak area with established calibration curves of each compound (fructose, glucose, sucrose, sorbitol, glucose 6-phosphate and tartrate). Solid-phase extraction (SPE) was used to separate organic acids from sugars on the samples, prior to HPLC analysis, as per instructions of the manufacturer.

2.6. Malate quantification

Malate quantification was performed recurring to the Malate Assay Kit (Sigma-Aldrich), using 30 mg of grape berry tissue homogenized in 500 µL of water for 30 min and then centrifuged at 10000 xg for 10 min. As per manufacturer instructions, the supernatant was mixed with malate assay buffer to a final volume of 50 µL per well. The reaction mixes were set up in a 96-well plate

according to the industrial kit directions and after 30 min of incubation in the dark, the absorbance of the samples was measured at 450 nm in a plate reader. The malate concentrations were determined using a calibration curve.

2.7. Enzyme assays

2.7.1. Total protein extraction

Grape berry total protein was extracted using a method described by Stoop and Pharr (1993) with several modifications. Sample powder was thoroughly mixed with extraction buffer in an approximately 1:1 (v/v) powder buffer ratio. Protein extraction buffer contained 50 mM Tris-HCl pH 8.9, 5 mM MgCl2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT) and 0.1% (v/v) Triton X-100. The homogenates were thoroughly mixed and centrifuged at 18000 xg for 20 min and the supernatants were maintained on ice and used for all enzymatic assays. Total protein concentrations of the extracts were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.7.2. Enzyme activity assays

Enzyme activity assays were performed as described by C. Sweetman and coworkers (2014) with some modifications. NAD-dependent cytosolic MDH activity was quantified as the rate of NADH oxidation at pH 6.0 (50 mM MES), in the presence of 5 mM oxaloacetate to ensure maximum velocity. NAD-dependent mitochondrial MDH activity was quantified as the rate of NAD reduction at pH 7.4 (MOPS) in the presence of 2 mM $MgCl_2$ and 10 mM malate to ensure maximum velocity. NAD-dependent mitochondrial ME activity was quantified as the rate of NAD reduction at pH 7.4 (50 mM MOPS) in the presence of 8mM $MnCl_2$ and 5 mM malate, to ensure maximum velocity.

Mannitol dehydrogenase (VvMTD) and sorbitol dehydrogenase (VvSDH) protein activity determination were carried out following Conde et al. 2015. VvMTD and VvSDH activity assays were performed at 37 °C, in a total reaction volume of 1ml. The reaction mixtures contained protein extract, 300 mM BIS-TRIS propane (pH 9.0), 1 mM NAD^+, and 200 mM d-mannitol or 200 mM d-sorbitol, for *VvMTD* or VvSDH activity respectively, to ensure maximum velocity. The reduction of NAD^+ was evaluated spectrophotometrically at 340 nm. All polyol oxidation reactions were initiated by the addition of the polyol. The SDH activity measurements in the direction of fructose reduction

were performed exactly like the mannitol oxidation assays, but using 200 mM fructose to start the reaction and 1 mM NADH as co-factor.

2.8. Statistical analysis

The results were statistically analyzed by Student's t test using Prism vs. 5 (GraphPad Software, Inc.). For each condition, statistical differences between mean values are marked with asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

RESULTS

3. Results

3.1. Phenological aspects of the grape berry during postharvest dehydration

As motioned in material and methods section, a slow dehydration process was applied to intact berry clusters for eleven days at 50°C and, consequently, changes in their phenological aspect were visible. With clear signals of dehydration, the grapes lose their initial appearance becoming darker and wrinkled as we can see in the Figure 5, and become noticeably sweeter. No fungal infection symptoms were visible.

A **B**

Figure 5. Grape berry clusters immediately after harvest (A) and after dehydration (B). Grapes berries were subjected to eleven days of a slow postharvest dehydration process at 50°C.

3.1.1. Water content

To evaluate the effect of dehydration in the water content, berry clusters' weight was measured before treatment (control), 5 days into the postharvest dehydration process and at the end of the treatment after 11 days. As reported in Figure 6, there was an increase of more than 3-fold in the dry weight-to-fresh weight ratio after day 11, when DW composed about 60% of total berry FW (Figure 6A). Inherently, water content progressively diminished from an initial 83% to just about 37% after postharvest dehydration (Figure 6B).

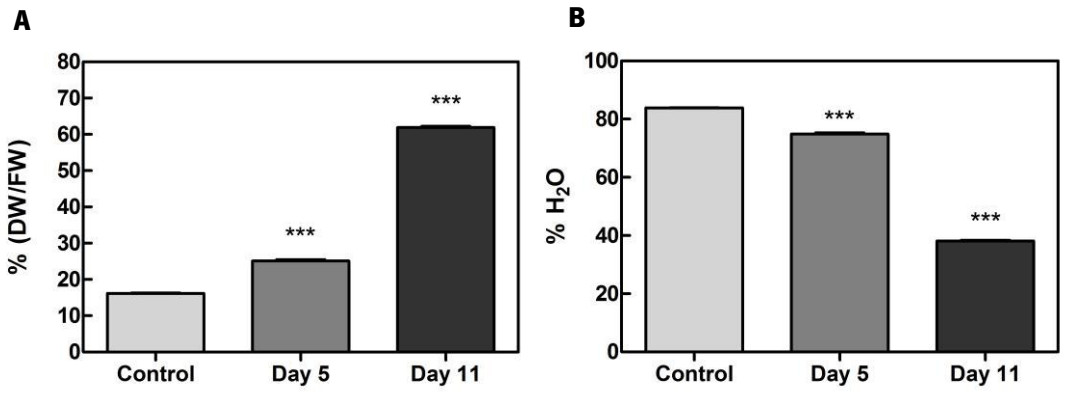

Figure 6. Effect of postharvest dehydration on water content of grape berries.

(A) Dry weight (DW)-to-fresh weight FW ratio expressed in percentage; **(B)** Percentage of berry water content; Measurements were made in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). Values are the mean ±SEM. Asterisks indicate statistical significance (Student's t-test; ***P<0.001).

3.1.2. pH

One of the strongest relationships between temperature and fruit quality occurs with grape berry acidity, whereby high temperatures reduce the concentration of organic acids (Sweetman et al. 2014). For that reason, pH values of grape berries subjected to dehydration process and without treatment (control) were measured. As shown in Figure 7, pH values of dehydrated grapes subjected to 5 days and 11 days of treatment were higher (4.1 and 4.0, respectively) when compared to the control (3.7).

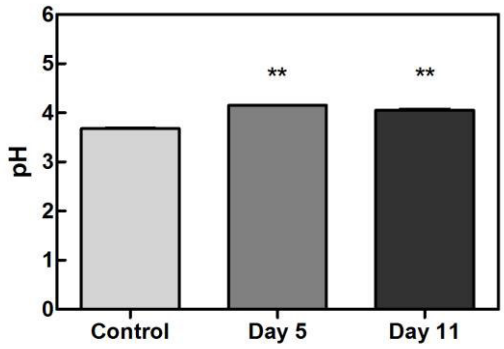

Figure 7. Effect of postharvest dehydration on the pH of grape berries. Measurement was made in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). Values are the mean ±SEM. Asterisks indicate statistical significance (Student's t-test; **P<0.01).

3.1.3. Chlorophylls and carotenoids

The quantification of photosynthetic pigments was accomplished according to Lichtenthaler and Buschmann (2005). The concentration of pigments, both carotenoids (Figure 8A) and total chlorophylls (Figure 8B), was lower in grape berries subjected to five (0.003 μg mg DW^{-1}) or eleven days (0.004 μg mg DW^{-1}) of dehydration process when compared to grapes without treatment (control) (0.01 μg mg DW^{-1}).

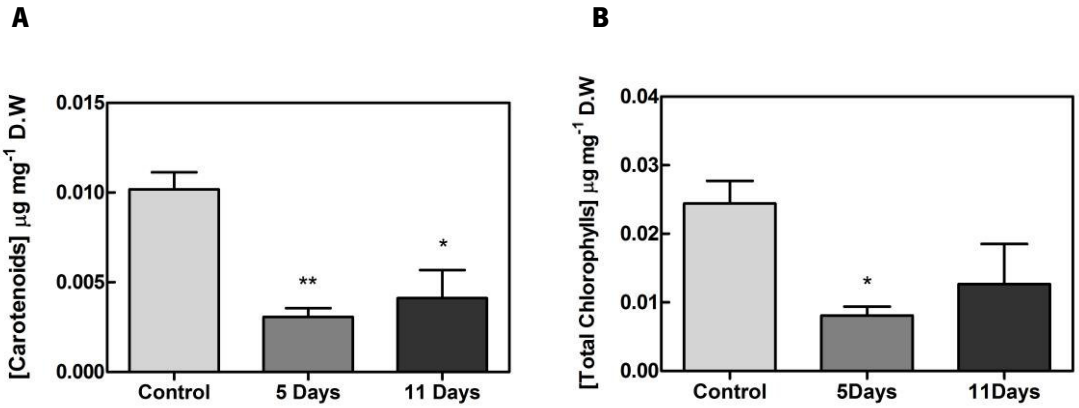

Figure 8. Effect of postharvest dehydration on the concentration of photosynthetic pigments in grape berries. Pigments were extracted from grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). The extracts were subsequently analyzed by spectrophotometry and pigment quantification was performed according to equations described in Material and Methods section. Concentration of carotenoids **(A)** and total chlorophylls **(B)** in samples extracts are expressed and μg per mg of DW. Values are the mean ±SEM. Asterisks indicate statistical significance (Student's t-test; *P<0.05; **P<0.01).

3.2. Modulation of aquaporin expression and water transport capacity

Aquaporins are membrane water channels that play critical roles in controlling cellular and tissue water movement as well as content (Fouquet et al. 2008). As we had significant water content variation in dried grapes, we assessed the gene expression of some plasma membrane and intracellular aquaporins.

3.2.1. Plasma membrane aquaporin

The abundance of the transcripts encoding for the plasma membrane intrinsic protein VvPIP2:1 was analyzed by real-time qPCR. As shown in Figure 9, *VvPIP2:1* expression in grapes

subjected to postharvest dehydration was extremely enhanced, particularly after the first five days of treatment (Figure 9), when it dramatically increased about 40000 fold. A subsequent dramatic decrease in *VvPIP2:1* transcripts at day 11 ensued.

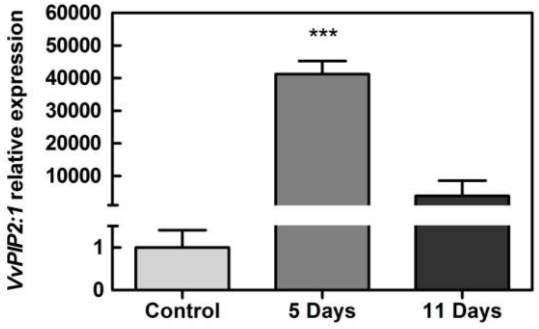

Figure 9. Effect of postharvest dehydration in the transcript levels of the plasma membrane intrinsic protein *VvPIP2:1* in grape berries. Gene expression analyses, by real-time qPCR, in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control); *VvPIP2:1* relative expression levels were obtained after normalization with the expression of the reference genes. *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; ***P<0.001).

3.2.2. Intracellular aquaporins

The gene expression of some intracellular aquaporins was also analyzed. Real-time qPCR revealed that the expression of the tonoplast intrinsic protein *(VvTIP1:2)* was also extremely increased by postharvest dehydration, predominantly after the first five days of treatment (Figure 10A). Also, the gene that encodes the endoplasmic reticulum (ER)-located grape berry small and basic intrinsic protein *(VvSIP1)* was up-regulated in dried grapes, particularly in 5 days dehydrated grapes (in about 6-fold) when compared to untreated berries (Figure 10B), however in an immensely lower magnitude than *VvTIP1:2* and plasma membrane AQPs overexpression.

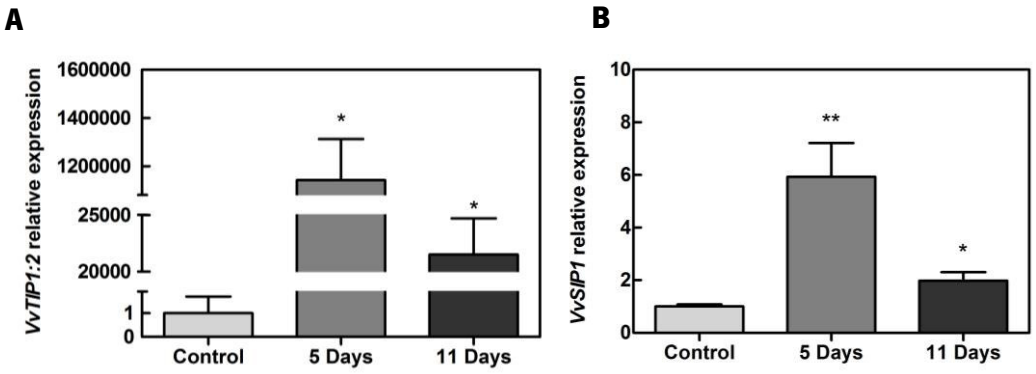

Figure 10. Effect of postharvest dehydration in the transcript levels of intracellular aquaporins in grape berries. (A) tonoplast intrinsic protein *VvTIP1:2;* **(B)** small and basic intrinsic protein gene expression analyses, by real-time q-PCR, in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control); *VvPIP2:1* relative expression levels were obtained after normalization with the expression of the reference genes. *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; *P<0.05; **P<0.01).

3.3. Modifications of sugar transport and metabolism in grape berries during postharvest dehydration

In order to evaluate possible changes, at berry level, in sugar transport capacity, concentration and metabolism in response to postharvest dehydration, metabolomic and transcriptional analysis approaches were adopted.

3.3.1. Transcriptional and metabolomic changes in sugar transport and metabolism

Sugar content in grape berries without treatment (control) and subjected to five or eleven days of dehydration process was quantified by HPLC. As shown in Figure 11A and Figure 11B, glucose and fructose concentration, respectively, were identical between all experimental conditions. Glucose concentration was estimated to be roughly 450 $\mu g\ mg^{-1}$ DW before postharvest dehydration, approximately 400 $\mu g\ mg^{-1}$ DW after 5 days of treatment and 420 $\mu g\ mg^{-1}$ DW at the end of it. Fructose concentration was estimated to be around 300 $\mu g\ mg^{-1}$ DW for all experimental conditions. Sucrose content was slightly decreased after 5 days of dehydration (by about 10%).

However, berries at five and eleven days of dehydration, had more glucose 6-phosphate along time of dehydration than control ones (Figure 11D).

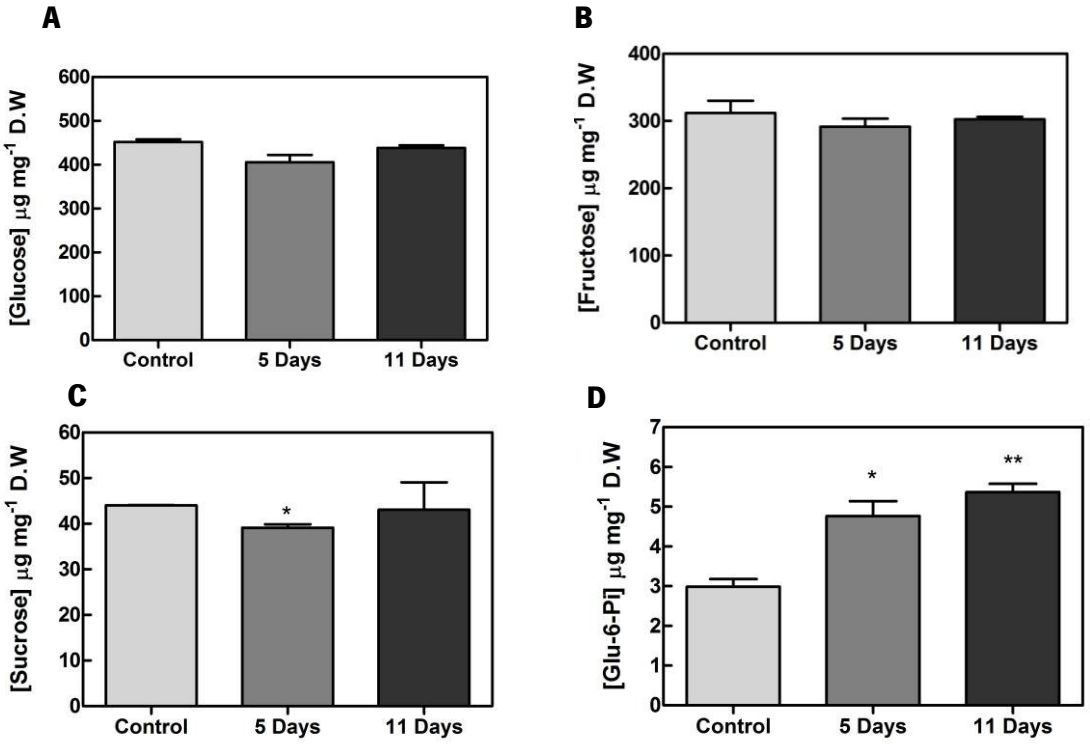

Figure 11. Effect of postharvest dehydration on the concentration of major sugars in grape berries. (A) Glucose; **(B)** fructose, **(C)** sucrose and **(D)** glucose 6-phosphate were quantified by HPLC in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; *P<0.05; **P<0.01).

To understand if sugar transport was modified due to dehydration process, the expression levels of some genes involved in this process were determined by real-time qPCR in grape berries without treatment (control) and subjected to five and eleven days of dehydration process. The plant SWEET family of sugar transporters is a recently identified protein family of sugar uniporters. They are typically bidirectional sugar transporters that mediate both high-capacity low-affinity uptake and efflux of sugar across the plasma membrane, catalyzing the transport of sucrose, fructose and/or glucose, depending on the transporter (Chong et al. 2014). *VvSWEET11* is highly homologous to Arabidopsis *AtSWEET11*, and exhibited, in dehydrated grapes, also a very strong overexpression comparatively to the control (Figure 12A). *VvSWEET15* transcripts were 3-fold higher in berries

subjected to five days of dehydration than in control (Figure 12B), while the expression of the plasma membrane hexose transporter *VvHT1* in the dehydrated berries was immensely increased relatively to the control (Figure 12C). The expression of the tonoplast monosaccharide transporter *VvTMT1* was also higher in berries subjected to postharvest dehydration process (Figure 12D). In agreement, the gene expression of cell wall invertase *VvcwINV* that catalyzes the conversion of apoplastic sucrose into glucose and fructose was also enhanced at both postharvest dehydration stages (Figure 12E).

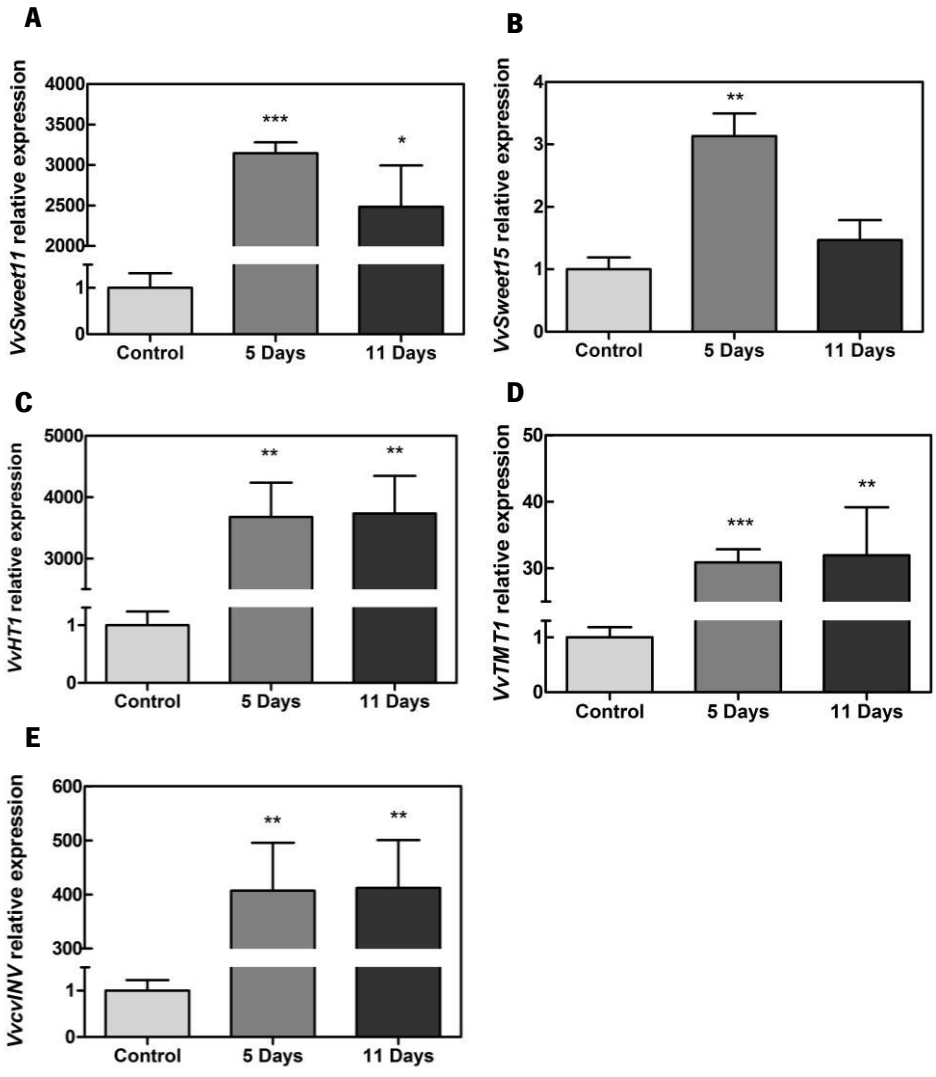

Figure 12. Effect of postharvest dehydration in the transcript levels of some grapevine sugar transporters in grape berries. Gene expression analyses, by real-time q-PCR, in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control); **(A)** SWEET11 transporter *(VvSWEET11)*; **(B)** SWEET15 transporter *(VvSWEET15)*; **(C)** Hexose transporter 1 *(VvHT1)*; **(D)**

Tonoplast monosaccharide transporter 1 *(VvTMT1);* cell wall invertase *(VvcwINV)* gene expression analyses. Gene relative expression levels were obtained after normalization with the expression of the reference gene *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; *P<0.05; **P<0.01; ***P<0.001).

3.3.2. Transcriptional, metabolomic, and enzyme activity changes in sugar alcohol transport and metabolism

To overcome the effects of abiotic stresses, plants have developed complex and active systems involving a wide range of biochemical and physiological processes. Polyols are important metabolites that frequently function as carbon and energy sources and/or osmoprotective solutes in some plants (Conde et al. 2015) and to evaluate possible changes in the sugar alcohol transport capacity, metabolism and concentration in response to postharvest dehydration, metabolomic and direct transcriptional analysis were adopted as strategies.

3.3.2.1. Polyol transport

VvPLT1 encodes a grapevine polyol transporter with the ability to also transport monosaccharides. Results show that *VvPLT1* transcripts abundance was severely and increasingly higher along the dehydration process in comparison with the control (Figure 13).

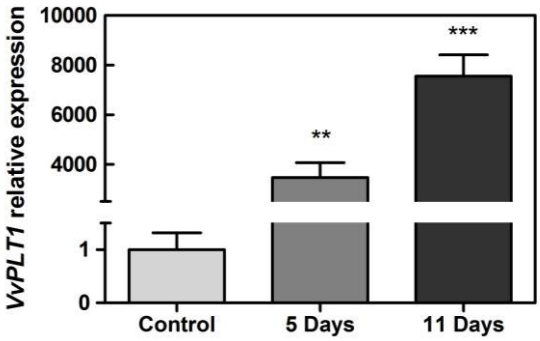

Figure 13. Effect of postharvest dehydration in the transcript level of the polyol transporter *VvPLT1.* Gene expression analysis, by real-time qPCR, of *VvPLT1* in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). *VvPLT1* relative expression levels were obtained after normalization with the expression of the reference gene *VvGAPDH.* One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; **P<0.01; ***P<0.001).

3.3.2.2. Sorbitol

Sorbitol content in grape berry tissues from all experimental conditions was quantified by HPLC-RI. As shown in Figure 14, sorbitol presence was only detected in grape berries subjected to postharvest dehydration reaching concentrations of up to 3.2 μg mg DW^{-1}.

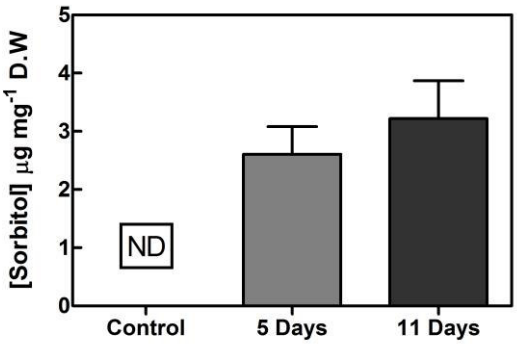

Figure 14. Effect of postharvest dehydration on the concentration of sorbitol in grape berries. Sorbitol was quantified by HPLC in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). Values are the mean ± SEM.

In grapevine, according to KEGG (Kanehisa and Goto, 2000) sorbitol is primarily and reversibly oxidized to sorbose or fructose in an reaction catalyzed by a sorbitol dehydrogenase (SDH). Transcriptional analyses by real-time qPCR shown that *VvSDH,* that encodes sorbitol dehydrogenase, was strongly up-regulated in grape berries subjected to postharvest dehydration (Figure 15), with roughly a 450-fold and 150-fold increase in expression at 5 and 11 days of treatment, respectively.

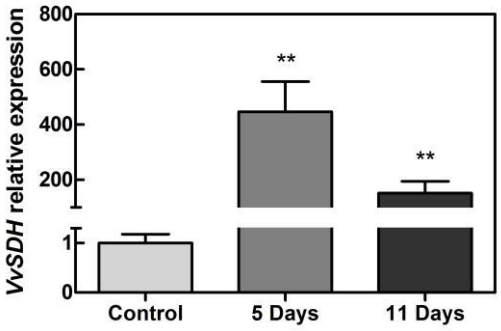

Figure 15. Effect of postharvest dehydration in the transcript level of *VvSDH* in grape berries. Gene expression analysis, by real-time qPCR, of *VvSDH* in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). *VvSDH* relative expression levels

were obtained after normalization with the expression of the reference gene *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; **P<0.01).

As sorbitol dehydrogenase reversibly oxidizes sorbitol to fructose we also measured the enzymatic activity of SDH, in both directions, in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). The results shown that in the direction of sorbitol oxidation (Figure 16A) the maximum velocity (V_{max}) of SDH was strongly reduced in dried grapes compared to control. In fact, its biochemical activity at a saturating concentration of sorbitol (200 mM) was diminished from an initial 2.1 μmol h^{-1} mg $protein^{-1}$ down to only about 0.3 and 0.4 μmol h^{-1} mg $protein^{-1}$ respectively at 5 and 11 days of postharvest dehydration. On the other hand, in the direction of sorbitol synthesis there is a slightly increase of the SDH activity in grapes subjected to five days of dehydration (Figure 16B), whereas no activity could be measured 11 days after beginning of the treatment.

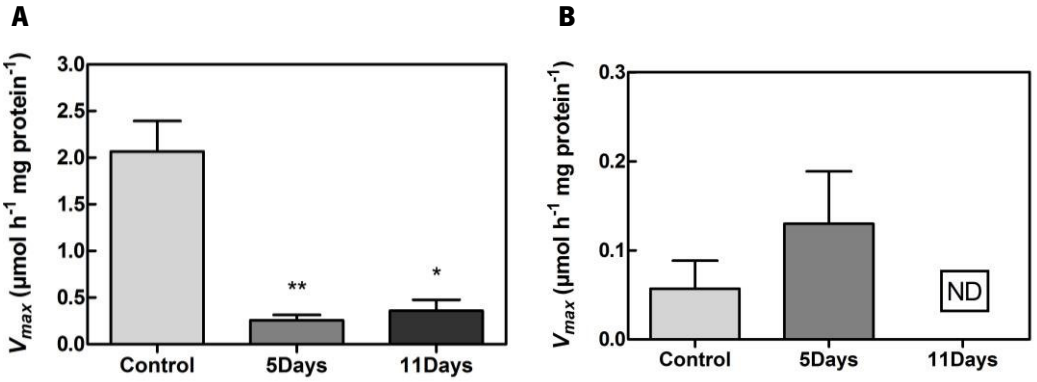

Figure 16. Effect of postharvest dehydration on sorbitol dehydrogenase (SDH) activity in grape berries. Sorbitol oxidation by SDH **(A)**; Sorbitol reduction by SDH **(B)**. Biochemical activity was measured in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). The assay was performed in triplicate. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; *P<0.05).

3.3.2.3. Mannitol

Mannitol is the most widespread polyol in nature and is accumulation is associated with crucial mechanism for salt/osmotic stress tolerance, for coping with heat stress-induced oxidative damage and excessive solar irradiance (Cimato et al. 2010; Melgar et al. 2009). The oxidation of mannitol is catalyzed by the cytosolic enzyme mannitol dehydrogenase (MTD) in a reversible but predominant reaction in which a molecule of fructose is formed (Conde et al. 2015). The expression of *VvMTD1* was only detected in the control, whereas the expression during dehydration was abolished to the point it was not detected (Figure 17A). A very similar observation was found when measuring the biochemical activity of MTD. In control berries, the maximum velocity of mannitol oxidation was approximately 1.3 μmol h^{-1} mg $protein^{-1}$, whereas it was totally undetectable in dehydrated grapes. (Figure 17B).

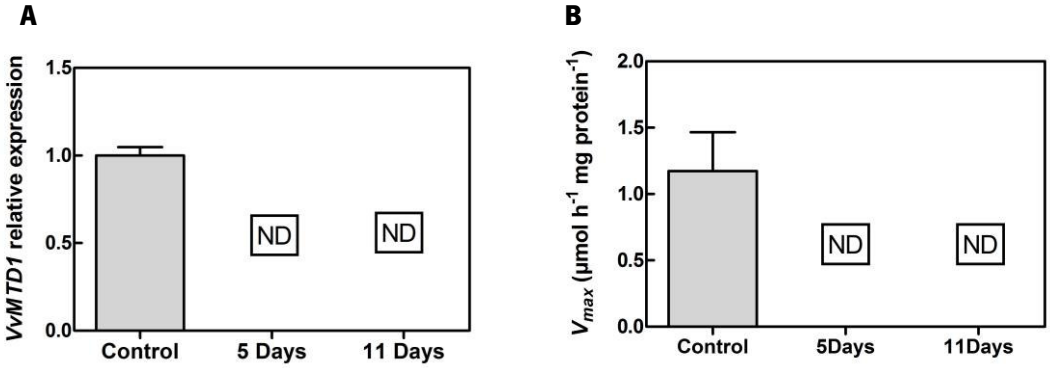

Figure 17. Effect of postharvest dehydration in the transcript level sof grapevine *VvMTD1* (A) and in mannitol dehydrogenase (MTD) activity (B) in grape berries. Gene expression analysis, by real-time qPCR, of *VvMTD1* **(A)** and biochemical activity of mannitol dehydrogenase **(B)** was measured in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). *VvMTD1* relative expression levels were obtained after normalization with the expression of the reference gene *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. The enzyme activity assay was performed in triplicate. Values are the mean ± SEM.

3.3.2.4. Galactinol

VvGOLS1 encodes a galactinol synthase, an enzyme responsible for the first step in the RFO biosynthetic pathway, synthesizing galactinol from UDP-D-galactose and *myo*-inositol. As shown in Figure 18, *VvGOLS1* transcripts abundance was substantially higher along the days of dehydration treatments in comparison with the control.

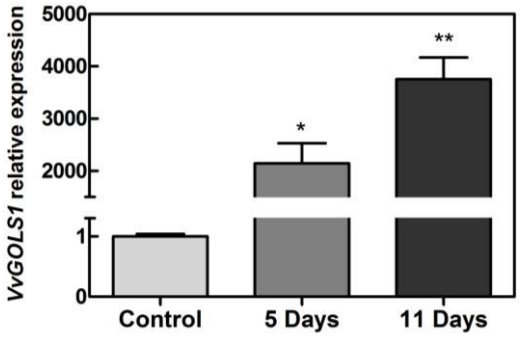

Figure 18. Effect of postharvest dehydration in the transcript levels of grapevine galactinol synthase VvGOLS1 in grape berries. Gene expression analysis, by real-time qPCR, of VvGOLS1 in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). VvGOLS1 relative expression levels were obtained after normalization with the expression of the reference gene VvGAPDH. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; *P<0.05; **P<0.01).

3.4. Modifications of organic acids metabolism in grape berries during postharvest dehydration

With the purpose of evaluating possible changes, at berry level, in organic acids metabolism in response to postharvest dehydration, metabolomic, transcriptional analysis and enzymatic activity determination approaches were used.

3.4.1. Quantification of the concentration of major organic acids

Tartrate content in grape berries subjected to five and eleven days of dehydration process and without treatment (control) was quantified by HPLC-RI. As reported in Figure 19A, tartrate content was strongly reduced in dehydrated grapes, reaching approximately 8 μg mg^{-1} DW after 5 days of dehydration, and, remarkably, less than 1 μg mg^{-1} DW after eleven days of treatment, so, much lower values than the initial ca. 13.5 μg mg^{-1} DW. As mentioned in materials and methods section, malate content was quantified using a specific malate quantification assay spectrophotometric kit. Similarly, malate content was lower in dried grapes compared to control (Figure 19B). The concentration of malate was of 1.5 μg mg^{-1} DW in untreated grapes, then lowering down to 0.5 μg mg^{-1} DW and 0.8 μg mg^{-1} DW in grapes dehydrated for 5 and 11 days, respectively.

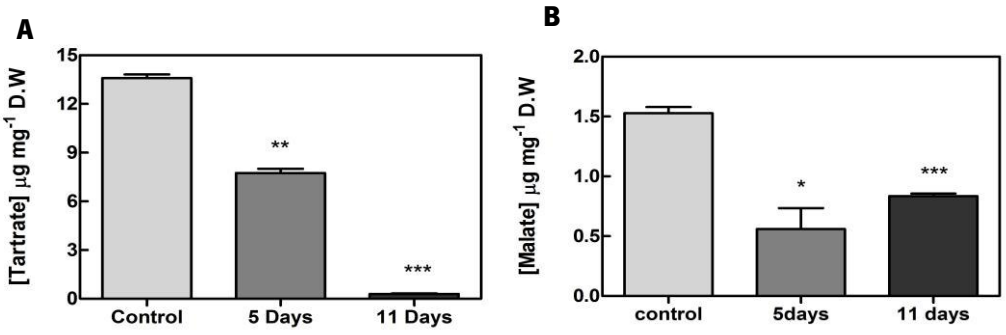

Figure 19. Effect of postharvest dehydration on the concentration of major organic acids (tartrate and malate) in grape berries. Tartrate was quantified by HPLC **(A)** and malate was quantified using a specific malate assay kit **(B)**, both in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; *P<0.05; **P<0.01; ***P<0.001).

3.4.2. Enzyme activity and transcriptional changes of molecular mechanisms involved in malate synthesis

Malate dehydrogenase (MDH) catalyzes a reversible reaction between OAA and malate, with higher affinities for NADH and OAA than for NAD^+ and malate, favoring the synthesis of malate (Sweetman et al. 2009). Analyses by real-time qPCR showed that the expression of *VvcytMDH* was only detected in control untreated grape berries, whereas 5 days and 11 days of dehydration resulted in a strong abolishment of gene expression to the point that it was totally undetected (Figure 20A). The total biochemical activity of cytosolic MDH (cytMDH) was also measured in all experimental conditions. Its maximum velocity was approximately 24 µmol h^{-1} mg $protein^{-1}$ in control berries and then severely decreased by about 10-fold after 5 and 11 days of postharvest dehydration (Figure 20B).

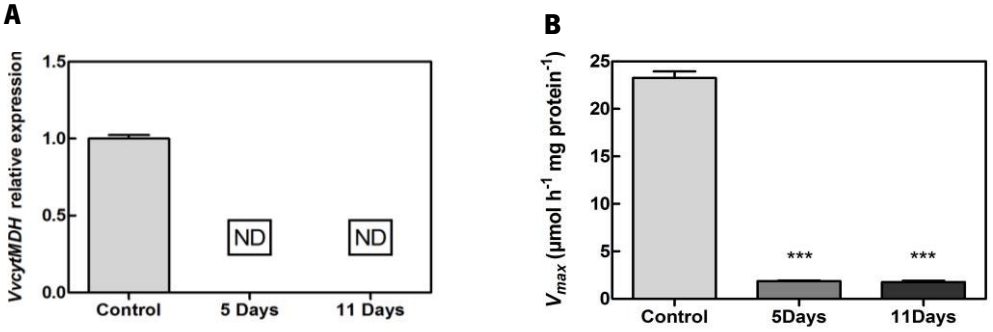

Figure 20. Effect of postharvest dehydration in the transcript levels of grapevine cytosolic malate dehydrogenase *(VvcytMDH)* (A) and in total cytosolic malate dehydrogenase activity (B) in grape berries. Gene expression analysis, by real-time qPCR, of *VvcytMDH* **(A)** and biochemical

activity of cytosolic malate dehydrogenase **(B)** was measured in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). *VvcytMDH* relative expression levels were obtained after normalization with the expression of the reference gene *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. The enzyme activity assay was performed in triplicate. Values are the mean ± SEM.

3.4.3. Enzyme activity and transcriptional changes of molecular mechanisms involved in malate degradation

Sweetman and coworkers (2009) suggested that mitochondrial malate dehydrogenase is predominantly involved in malate degradation. Analyses by real time qPCR showed that *VvmytMDH* was strongly upregulated in dehydrated grapes (Figure 21A). The total enzymatic activity of mitochondrial malate dehydrogenase was also measured, but only detected in grapes subjected to postharvest dehydration with maximum velocities of 0.5 and 0.25 μmol h^{-1} mg $protein^{-1}$ respectively after 5 and 11 days of dehydration (Figure 21B).

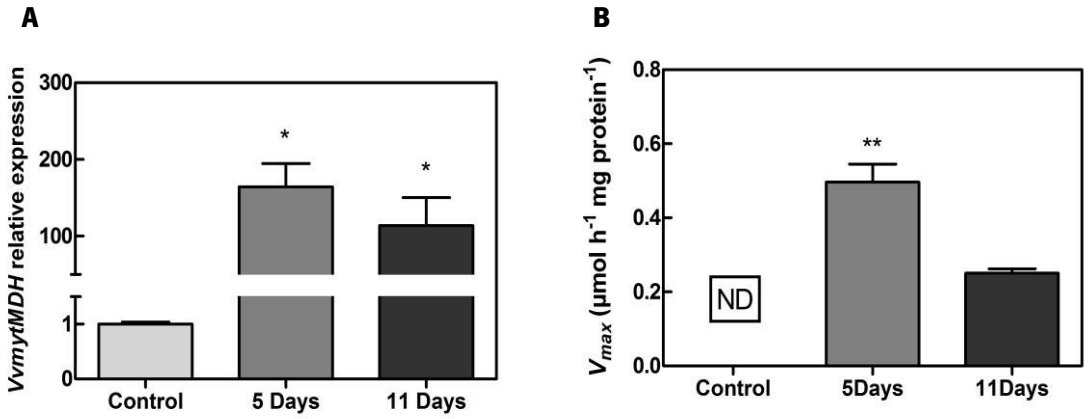

Figure 21. Effect of postharvest dehydration in the transcript levels of grapevine mitochondrial malate dehydrogenase *(VvmytMDH)* (A) and in total mitochondrial malate dehydrogenase activity (B) in grape berries. Gene expression analysis, by real-time qPCR, of *VvmytMDH* **(A)** and biochemical activity of mitochondrial malate dehydrogenase **(B)** was measured in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). *VvmytMDH* relative expression levels were obtained after normalization with the expression of the reference gene *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. The enzyme activity assay was performed in triplicate. Values are the mean ± SEM.

In grapes, NADP-dependent malic enzyme (NADP-ME) is a key intervenient in malate degradation as it reversibly, but favourably, catalyzes pyruvate and NADPH synthesis using malate and $NADP^+$ as substrates (Sweetman et al. 2009). Real time qPCR studies demonstrated that *VvmytME* transcript abundance was immensely higher in grapes subjected to 5 and 11 days of dehydration than in the control (Figure 22A). We also measured the enzymatic activity of NADP-ME and but were only able to was detect it in grapes subjected to five days of dehydration (Figure 22B), with a maximum velocity of nearly 2.5 μmol h^{-1} mg $protein^{-1}$ respectively.

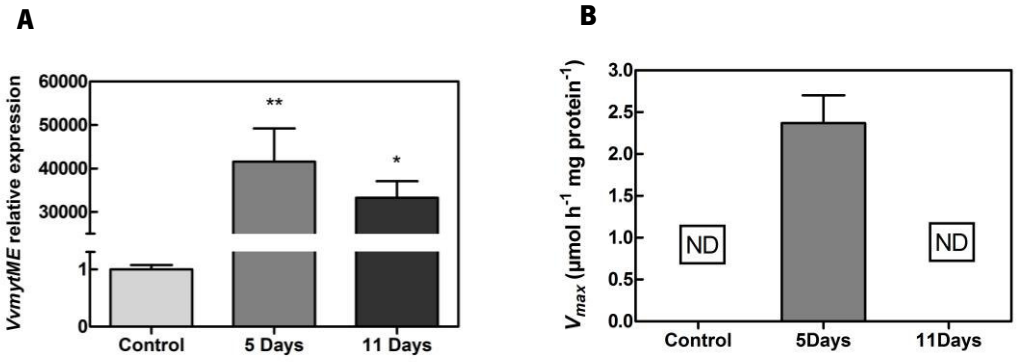

Figure 22. Effect of postharvest dehydration in the transcript level of grapevine mitochondrial malic enzyme *(VvmytME)* (A) and in total mitochondrial malic enzyme activity (B) in grape berries. Gene expression analysis, by real-time qPCR, of *VvmytME* **(A)** and biochemical activity of malic enzyme **(B)** was measured in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). *VvmytME* relative expression levels were obtained after normalization with the expression of the reference gene *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. The enzyme activity assay was performed in triplicate. Values are the mean ± SEM.

3.5. Modifications of secondary metabolism during postharvest dehydration

Secondary metabolism has an important role in plant defense against biotic and abiotic factors and is extremely impactful in wine production because it is responsible for phenolic and volatile compound synthesis in grape cells. Therefore, we also investigated secondary metabolism alterations induced by postharvest dehydration.

The enzyme 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase is the first enzyme of the shikimate pathway, which is responsible for the biosynthesis of the amino acids phenylalanine, tyrosine, and tryptophan, controlling the amount of carbon entering the in pathway

(Maeda and Dudareva, 2012). The expression of *VvDAHPS1* was up-regulated in about 30-fold in grapes berries subjected to dehydration (Figure 23).

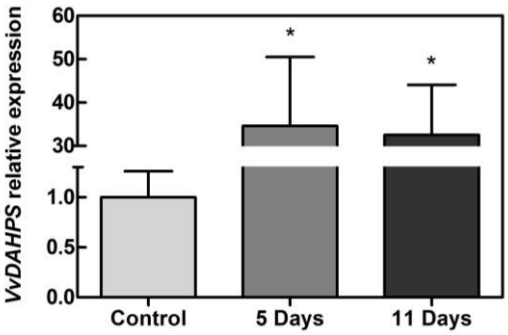

Figure 23. Effect of postharvest dehydration in the transcript levels of *VvDAHPS1* in grape berries. Gene expression analysis, by real-time qPCR, of *VvPDAHPS1* in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). *VvDAHPS* relative expression levels were obtained after normalization with the expression of the reference gene *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; *P<0.05).

3.5.1. Transcriptional changes in phenylpropanoids pathway

Phenylpropanoids are indicators of a wide range of plant responses towards biotic and abiotic stresses (La Camera et al. 2004). The phenylpropanoids pathway is characterized by the redirection of the carbon flow from primary metabolism and the enzyme phenylalanine ammonia-lyase (PAL) catalyzes the first step of this pathway. By The expression studies on one PAL gene showed that *VvPAL1*, transcripts abundance was increasingly higher along the longevity of the dehydration treatment in comparison with the control (Figure 24).

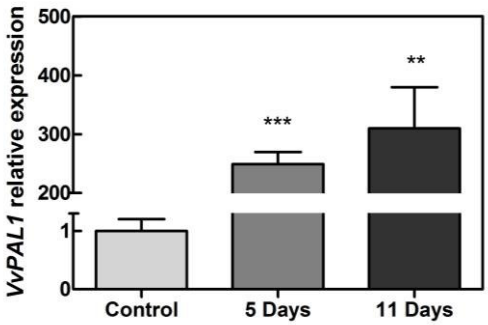

Figure 24. Effect of postharvest dehydration in the transcript levels of grapevine phenylalanine ammonia-lyase 1 (VvPAL1) in grape berries. Gene expression analysis, by real-time

qPCR, of VvPAL1 in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). VvPAL1 relative expression levels were obtained after normalization with the expression of the reference gene VvGAPDH. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; **P<0.01; ***P<0.001).

3.5.2. Transcriptional changes in stilbene pathway

Stilbenes are naturally-occurring compounds found in edible plants like *Vitis* and stilbene biosynthesis increases upon pathogen infection and in response to abiotic stresses. *VvSTS1* encodes stilbene synthase (STS), that is the key enzyme in stilbene biosynthesis (Bavaresco et al. 2009). Results demonstrated that *VvSTS1* transcripts abundance was higher in comparison with the control (Figure 25).

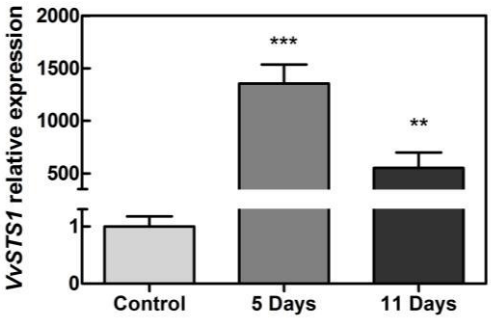

Figure 25. Effect of postharvest dehydration in the transcript levels of grapevine stilbene synthase 1 (VvSTS1) in grape berries. Gene expression analysis, by real-time qPCR, of VvSTS1 in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). VvSTS1 relative expression levels were obtained after normalization with the expression of the reference gene VvGAPDH. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; **P<0.01; ***P<0.001).

3.5.3. Transcriptional changes in flavonoids pathway – flavonol biosynthesis

The flavonoid pathway is initiated by the action of chalcone synthase (CHS). By real-time qPCR it was observed that *VvCHS1* was highly expressed in grapes subjected to postharvest dehydration, being particularly and tremendously up-regulated after the first five days of treatment (Figure 26A). Flavonol synthase (FLS) is the first enzyme of the flavonol biosynthetic branch of the flavonoid pathway. The expression of *VvFLS1* was progressively up-regulated along the dehydration treatment (Figure 26B).

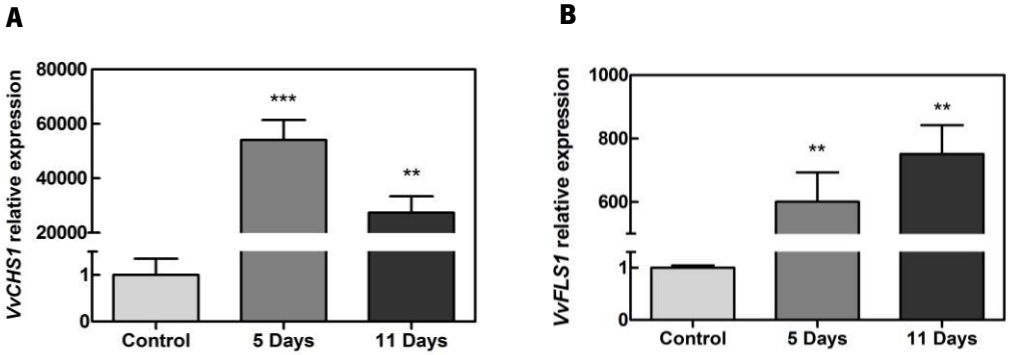

Figure 26. Effect of postharvest dehydration in the transcript levels of two genes involved in the flavonoids pathway in grape berries. Gene expression analysis, by real-time qPCR, of *VvCHS1* **(A),** and Flavonol synthase (*VvFLS1*) **(B)** in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). *VvCHS1* and *VvFLS1* relative expression levels were obtained after normalization with the expression of the reference gene *VvGAPDH*. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; **P<0.01; ***P<0.001).

3.5.4. Concentration of total phenolics and anthocyanins

Since molecular changes in several steps of secondary metabolic pathways were observed, the total amount of phenolic compounds and anthocyanins were quantified. The concentration of total anthocyanins of dried grapes was just about the same as that in control berries (Figure 27A). On the other hand, berries subjected to eleven days of postharvest dehydration had approximately less 80% of phenolic compounds compared to control (Figure 27B), while the reduction after 5 days of treatment was of about one third of the initial concentration.

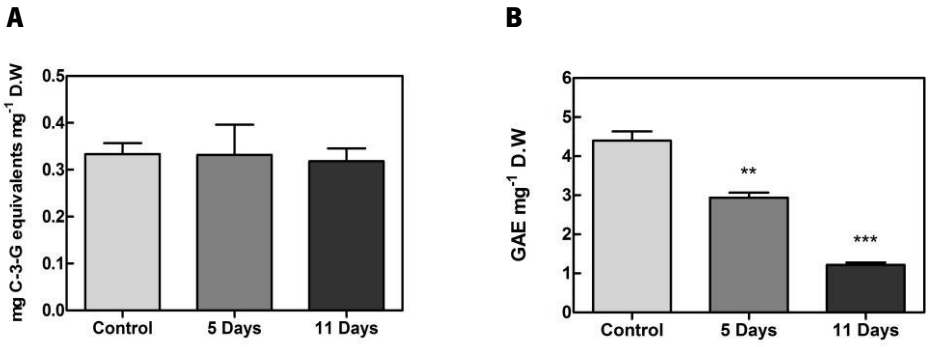

Figure 27. Effect of postharvest dehydration on anthocyanins (A) and total phenolics (B) concentration in grape berries. Anthocyanin concentration **(A)** is represented as mg mL^{-1} of cyanidin-

3-glucoside (C-3-G) equivalents per mg dry weight (DW) and total phenolic compounds concentration **(B)** is represented as μg mL^{-1} of gallic acid equivalents (GAE) per mg of dry weight in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). Asterisks indicate statistical significance (Student's t-test; **P<0.01; ***P<0.001).

3.5.5. Degradation of phenolics

There is evidence that elevated temperatures have a negative impact on the color and phenolic contents of some red berry grapevine varieties, thus affecting the final color of some wines (Mori et al. 2007) . As some recent studies indicate that peroxidases are the main candidates for anthocyanin degradation (Movahed et al. 2016), we decided to analyze changes in transcript levels of peroxidase 31 *(VvPRX31)*, that encodes a referenced candidate for degradation of phenolics/anthocyanins. Results indeed demonstrated that *VvPRX31* transcripts abundance was extremely higher in comparison with the control and shown a superior up-regulation (up to 140000-fold after 5 days of dehydration) than the other secondary metabolism targets (Figure 28).

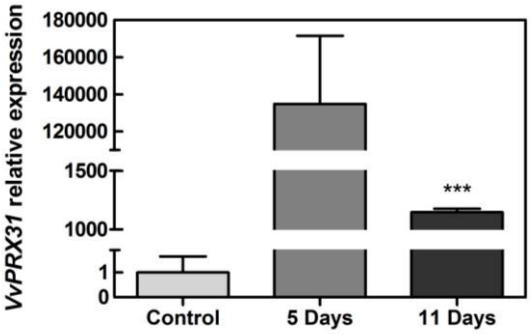

Figure 28. Effect of postharvest dehydration in the transcript levels of grapevine peroxidase 31 (VvPRX31) in grape berries. Gene expression analysis, by real-time qPCR, of VvPRX31 in grape berry tissues subjected to five and eleven days of dehydration process and without treatment (control). VvPRX31 relative expression levels were obtained after normalization with the expression of the reference gene VvGAPDH. One PCR run with triplicates was performed for each tested mRNA. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; ***P<0.001).

DISCUSSION

4. Discussion

Berries for sweet dessert wines (e.g. Recioto, Vin Santo) and dry fortified wines (e.g. Amarone) suffer a phase of postharvest dehydration in which cellular metabolism is significantly changed. There are already some studies of metabolic changes in Malvasia, Trebbiano and Sangiovese grapes during postharvest drying (Zamboni et al. 2008) but the knowledge at the molecular level is fairly scarce. The aim of this work was to go further, so we thoroughly investigated particular metabolic differences triggered during this process. Several molecular mechanisms were assessed by a combination of molecular and biochemical approaches, from water and solute transport mechanisms to primary and secondary metabolism with an emphasis on the metabolism of organic acids and secondary metabolites, due to their relevance in berry/wine flavor; and polyols, due to their involvement in water deficit stress tolerance. We also tried to understand the molecular response of sugar transporters, which are involved in sugar allocation from berry apoplast into the cells, to the postharvest dehydration process, as well as that of aquaporins and polyol transporters.

4.1. Postharvest dehydration leads to morphological changes in grape berry and enhancement of water transport capacity

It is reported that in postharvest berries, the rate of water loss induces cell wall enzyme activity, increases respiration and ethylene production, and causes the loss of volatiles (Hsiao, 1973; Bellincontro et al., 2004; Costantini et al., 2006). Subjected to eleven days of 50°C, in order to mimic some routinely used grape dehydration industrial procedures, our grapes suffered major changes in their structure and texture, becoming darker and wrinkled, exhibiting typical dried fruit appearance, suggesting a clear change in superficial cell architecture, reduction of intercellular space, and cell squeezing (Ramos et al. 2004). After eleven days subjected to 50°C, berries lost almost 40% of weight in water. Aquaporins are membrane water channels that play critical roles in controlling the water content of cells and tissues and transcriptional analyses by real-time qPCR showed that the expression of all genes tested that encode aquaporins, *VvPIP2;1, VvTIP1;2 and VvSIP1* was strongly up regulated in dried grapes, principally during the first five days of treatment. Aquaporins are water channel proteins that allow rapid and selective transport of water across membranes, which would presumably also affect water movement into the cell (Seymour et al. 2013). Their strong up-regulation suggests a possible mechanism of cell defense against

dehydration and loss of water, eventually by enhancing the capacity of water uptake from apoplast and water distribution between mesocarp cells during postharvest dehydration.

4.2. Postharvest dehydration improved sugar transport capacity in berries

Sugar content is quite relevant from a berry quality point of view and, therefore, for winemaking since it determines alcohol percentage after fermentation and influences the sensorial proprieties of the wine, especially in fortified ones obtained from dehydrated grapes.

Transcriptional analysis by real-time qPCR revealed that all sugar transporter genes studied, *VvHT1* and *VvTMT1* (plasma membrane and tonoplast high-affinity monosaccharide transporters, respectively), *VvSWEET11* and *VvSWEET15* (putative high-capacity bidirectional transporters of sucrose and hexoses), were all highly up-regulated in grapes subjected to postharvest dehydration, providing further evidence for a general stimulation of post-phloem sugar transport capacity from berry apoplast into cells, intracellularly from cytosol to the vacuole, and quite possibly also suggesting an increased capacity at the molecular level for sugar redistribution between berry cells due to the putative bidirectional transport capacity of SWEET transporters. Additionally, the gene coding for cell wall invertase (cwINV) was also more expressed in dried grapes, suggesting an increase of apoplastic sucrose conversion into glucose and fructose. This is in agreement with the slight decrease observed in the sucrose concentration from the initial condition before dehydration to grapes subjected to 5 or 11 days of dehydration. These results are supported by similar observations in postharvest withering in grape (Zamboni et al. 2008), where hexose transporters located in the plasma membrane were up-regulated in response to off plant withering.

However, the apparent enhancement of sugar transport capacity inside the berry did not translate into increased sugar concentration in berries subjected to postharvest dehydrated. In fact, fructose and glucose concentrations determined by HPLC were similar in dried and control grapes, while sucrose concentration was slightly lowered after 5 days of postharvest dehydration. On the other hand, interestingly, the amount of glucose-6-posphate almost doubled in grapes subjected to postharvest dehydration, compared to control. In grapevine, stress conditions sometimes increase the production of secondary metabolites among other responses, which may require additional glucose-6-phosphate to increase the availability of reducing power via the oxidative pentose phosphate pathway. A recent study (Noronha et al. 2015) suggested that the increase in the activity of putative glucose-6-phosphate transporters could drive this metabolite to the biosynthesis of

secondary metabolites in stress conditions. Taking our findings together, it is perfectly plausible that sugars were used to feed other metabolic pathways instead of being just stored in the vacuole of berry cells, such as, for instance, glucose-6-phosphate biosynthesis, an hypothesis reinforced by the combination of increased *VvcwINV* transcripts and slight decrease in sucrose concentration (at 5 days) but roughly similar concentration of glucose and fructose despite strongly increased monosaccharide transport capacity evidenced by upregulation of *VvHT1* and *VvSWEET* transporters.

4.3. Enhancement of polyol accumulation in grape berries subjected to postharvest dehydration

As mentioned in the introduction section, polyols, like sorbitol and mannitol, are important metabolites that usually function as carbon sources and/or osmoprotective solutes in some plants. Conde and co-workers (2015), recently suggested that *VvPLT1* is an H^+-dependent polyol transporter transcriptionally regulated by environmental challenges, like water deficit and dehydration. Analyses by real-time qPCR shows that *VvPLT1* was hugely up-regulated in dried gapes and the reported high affinity of this transporter for sorbitol and mannitol, clearly suggest that postharvest dehydration stimulated the capacity for accumulation of polyols into berry cells as a molecular response. Quantification by HPLC showed that sorbitol was only detected and, therefore, accumulated in grape berries subjected to postharvest dehydration. Also transcriptional analyses unveiled a stimulatory effect of dehydration stress in *VvSDH* transcripts. In parallel, enzymatic activity of SDH was increased after 5 days of postharvest dehydration in the direction of sorbitol synthesis, whereas, simultaneously its biochemical activity was severely inhibited in the direction of sorbitol oxidation in postharvest dehydrated grapes. This biochemical feature may very well be at least in part responsible for the accumulation of sorbitol in dehydrated grape berries. Mannitol was not detected in our HPLC analysis, however, transcriptional analysis of *VvMTD1*, a gene encoding for a mannitol dehydrogenase, was only expressed in control berries. In agreement, the total mannitol oxidation activity of MTD was too only detected in untreated berries, suggesting a dramatic abolishment of mannitol oxidation rate in dehydrated grapes. All these evidences strongly suggest that grape berries may accumulate and use polyols as osmoprotectants during the process of postharvest dehydration as a response triggered to try to maintain cell and tissue homeostasis.

Raffinose family oligosaccharides (RFOs), like raffinose and stachyose, are involved in protection against abiotic stress, and galactinol is a polyol that acts in their biosynthetic pathway as a galactosyl donor for RFO synthesis (Pillet et al. 2012; Conde et al. 2015). The expression of a galactinol synthase gene *(VvGOLS1)* was strongly enhanced in postharvest dehydration conditions, suggesting that it was a molecular adaptation in order to provide more galactinol to possibly act simultaneously as an osmoprotective and as an intermediary in the synthesis of raffinose (Nishizawa-Yokoi et al. 2008), in a defensive mechanism against dehydration.

4.4. The concentration of major organic acids decrease during postharvest dehydration as result of molecular changes in organic acid metabolism

Organic acids represent a key component of fruit organoleptic quality and their content is significantly influenced by temperature. One of the clearest relationships between temperature and fruit quality occurs with grape berry acidity, whereby high temperatures reduce the concentration of organic acids (Sweetman et al. 2014). Quantifications of malate and tartrate concentration in berries showed exactly that tendency as the content of these two main organic acids present in grape berries was extremely reduced in grapes subjected to postharvest dehydration. Malate, in grape berries, is synthetized from phosphoenolpyruvate (PEP) and cytosolic malate dehydrogenase (MDH) (Sweetman et al. 2009). Analyses by real-time qPCR demonstrated an absence of detectable expression of *VvcytMDH* in grapes subjected to dehydration treatments very well correlated with near absent biochemical activity of cytosolic malate dehydrogenase in dried grapes when compared to control berries. This suggests that the activity of this enzyme, responsible for malate synthesis, is arrested in response to postharvest dehydration. In parallel, the dehydration process severely increased the transcript abundance and biochemical activity of enzymes that preferentiality degrade malate, such as malic enzyme (ME) and mitochondrial MDH, which is probably a main cause for the net malate loss. Malic enzyme-catalysed conversion of mitochondrial malate to pyruvate provides NADH to the mitochondrial electron transport chain and acetyl-CoA (from pyruvate) to the tricarboxylic acid (TCA) cycle or the Krebs cycle. Malate can also enter the TCA cycle directly through the activity of mitochondrial MDH. There are reports that increased ME activity may facilitate increased respiration rates in heated grapevine clusters and malate can be used as a fuel source during ripening. Sweetman and co-workers (2014) added that observed increases in numerous amino acids, typically those derived from pyruvate, oxaloacetate and α-

ketoglutarate, indicate a change in TCA cycle regulation that could be a result of the observed increase in malic enzyme activity and organic acids degradation. Our findings suggest that indeed organic acids like malate could be possibly being used as carbon/energy source instead of sugars during the postharvest dehydration process.

4.5. Phenolic compounds are degraded during postharvest dehydration in grape berries despite apparently increased synthesis capacity

Phenolic compounds have great relevance in wine sensorial properties. Besides flavour properties, volatile phenolics contribute to aromas in a wine, while other phenolics have a significant role in plant defense mechanisms against biotic and abiotic factors.

Transcriptional analyses focused on secondary metabolism by real-time qPCR were performed and berry dehydration appeared to induce phenylpropanoid pathway, as suggested by the up-regulation of the *VvPAL1*, which creates precursors for many different groups of phenolic compounds. *VvSTS1* transcript abundance also stimulated by postharvest dehydration, suggesting increased stilbene production ability. Stilbenes are synthesized constitutively in seeds and are also produced in berry skin during development, and in response to biotic or abiotic stresses (Soleas et al. 1997). Significant resveratrol accumulation occurs during the postharvest drying of berries of many grape cultivars, and this has already been linked to the high-level expression of stilbene synthase (STS) (Versari et al. 2001). Among the up-regulated secondary metabolism-related transcripts in grapes subjected to postharvest dehydration were one chalcone synthase *(VvCHS1)* and a flavonol synthase *(VvFLS1)* gene. The transcriptional profile of the first gene suggests an activation of the first step in the flavonoid pathway during the dehydration process, while the transcriptional enhancement of the second one indicates a probable activation of the flavonol-synthetic branch of this pathway.

In spite of the enhancement of transcription of some key molecular players in biosynthetic pathways of secondary metabolism, the concentration of total phenolics in grapes subjected to postharvest dehydration was clearly reduced along treatment period. This observation is in agreement with the reported by Zamboni and co-workers (Zamboni et al. 2008), and appears to be a common occurrence in grape berry postharvest dehydration. Very little is known, however, regarding phenolics degradation in grape berry cells. As already mentioned, *VvPRX31,* encodes a referenced candidate for degradation of phenolics/anthocyanins and interestingly, *VvPRX31* was

extremely overexpressed in dehydrated grapes. This overexpression was in fact much higher than that observed in our targeted genes involved in secondary metabolic pathways that lead to phenylpropanoids, stilbenes and flavonoids synthesis. This suggests that *PRX31* could be one of the peroxidases involved in phenolic degradation in berry tissues exposed to postharvest dehydration, and that the triggered phenolic degradation in response to postharvest dehydration was actually far superior than a possible increase in phenolic synthesis, thus accounting for a strong decrease in phenolic content during dehydration. Moreover, a recent study also suggests the involvement of PRX31 in anthocyanin degradation in berry tissues exposed to heat stress (Movahed et al. 2016), despite no differences in the anthocyanin concentration were here observed.

In the cases when no enzymatic activity was measured, some cautious is, however, necessary in the assumption that an increased gene expression is per se a clear cut evidence that its corresponding molecular step is necessarily stimulated. However, both the great magnitude of the increase in the expression in most cases as well as the good correlation between gene expression of a given isoform and the total biochemical activity of the corresponding enzyme, that is the final result of all isoforms appear to reinforce the predictive value of a strong gene upregulation as evidence for increased activity of its corresponding metabolic step. Indeed, in the cases when both gene expression and biochemical activities were measured, it is tempting to assume there is strong transcriptional regulation occurring in the observed biochemical modifications.

In summary, we applied several molecular and biochemical techniques that clearly showed us that, after detachment from the mother grapevine, fruit tissues remain metabolically active. Our study showed that metabolism of harvested berries was strongly influenced in response to dehydration treatments. Typical dehydration treatments lead to an enhancement of water transport capacity via upregulation of aquaporin gene expression as molecular response, but further analyses are required to better understand the reason behind that up-regulation in water transporters.

Postharvest dehydration provoked an improvement in sugar transport capacity into the cells as demonstrated by the increase in transcripts of sucrose and monosaccharide transporters putatively involved in post-phloem transport in berry. Moreover, grape berries from postharvest dehydration treatments had an enhanced polyol biosynthesis, supporting their role as osmoprotectants and suggesting their accumulation as a defensive mechanism triggered during

postharvest dehydration. Additionally, postharvest dehydration severely affected the metabolism of organic acids in grape berries, as significant transcriptional and biochemical modifications in key enzymes lead to organic acid (malate and tartrate) degradation and synthesis inhibition. Grapes subjected to postharvest dehydration had an enhanced phenolic degradation that resulted in less concentration of phenolics. Assuming that changes in gene expression pattern are mirrored by metabolic changes, our data also represent robust evidence for the marked effects of postharvest water loss on metabolites synthesis/consumption and composition. The observed changes undoubtedly affect organoleptic characteristics of wines and other products resulting from typical postharvest dehydration processes.

The approaches used in this study could also be performed to evaluate the influence of postharvest dehydration on aroma biosynthesis, other important metabolic pathways associated with berry quality. Also the detection of sorbitol in grape berries in response to water-deficit stress, together with its genetic and molecular determinism, deserve further investigations as, while *in planta* it may have an important application in the improvement of grapevine practices and water use efficiency, in grape postharvest dehydration could be a factor of increased nutraceutical value due to the antioxidant properties of polyols.

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